Investigation of novel candidates for targeted therapy in melanoma

by

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2. LIST OF PUBLICATIONS

I. Magnussen GI, Ree Rosnes AK, Shahzidi S, Dong HP, Emilsen E, Engesaeter B, Flørenes VA. Synthetic retinoid CD437 induces apoptosis and acts synergistically with TRAIL receptor-2 agonist in malignant melanoma. Biochem Biophys Res Commun 2012 Apr 13;420(3):516-22.

II. Magnussen GI, Holm R, Emilsen E, Rosnes AK, Slipicevic A, Flørenes VA. High expression of Wee1 is associated with poor disease-free survival in malignant melanoma: potential for targeted therapy. PLoS One 2012;7(6):e38254.

III. Magnussen GI, Emilsen E, Fleten KG, Engesaeter B, Nähse-Kumpf V, Fjaer R, Slipicevic A, Flørenes VA. Combined inhibition of the cell cycle related proteins Wee1 and Chk1/2 induces synergistic anti-cancer effect in melanoma. Submitted manuscript.

3. AIMS OF STUDY

Malignant melanoma is one of the most increasing forms of cancer, in addition to being notoriously resistant to therapy once it has reached an advanced stage. New treatment alternatives have emerged in recent years and have led to improved overall survival for some patients, however all are associated with adverse effects and development of resistance. The search for new treatment strategies is thus still of importance.

The aims of this thesis were to:

I. Study the anti-tumour effect of the synthetic retinoid, CD437, in melanoma cell lines, and to evaluate possibilities for combinational treatment.

II. Examine the expression of the cell cycle regulatory protein, Wee1, in melanoma specimens, and analyze its relationship to clinicopathological parameters and patient survival.

III. Investigate the potential of Wee1 as a target for therapy in melanoma, alone or in combination with the cell cycle regulatory proteins Chk1/2.

4. INTRODUCTION

4.1 Cancer

Over time, normal cells can transform into cancerous by acquiring a series of mutations which includes activation of oncogenes (*"a gene that, when activated by mutation, increases the selective growth advantage of the cell in which it resides"*), inactivation of tumour suppressor genes ("*a gene that, when inactivated by mutation* (or alterations such as methylations)*, increases the selective growth advantage of the cell in which it resides"*) and malfunction of DNA-repair genes [1,2]. Although the average human tumour cell harbours many mutations, the majority of cancers are caused by only two to eight 'driver mutations' that are usually acquired sequentially over time [2]. Tumourigenesis, the step-wise process in which a normal cell is transformed, allows it to undergo uncontrolled cell division that leads to the formation of a malignant mass that can ultimately spread to other parts of the body. For this process to occur, the cell has to acquire certain traits that includes the ability to sustain proliferative signalling, evade growth suppressors, avoid immune destruction, enable replicative immortality, activate invasion and metastasis, induce angiogenesis, resist cell death and deregulate cellular energetic. Additionally, tumour promoting inflammations, as well as genetic instability, are enabling characteristics suggested to underlie the mentioned hallmarks and drive tumour progression (Figure 1) [3]. The tumour microenvironment has also been found to contribute to the hallmark capabilities and thereby the formation of cancer [4]. The tumourigenic process leads to genetic heterogeneity among the cells within a tumour, which can impact the therapeutic response. As a disease, cancer is only seconded by cardiovascular disease as the leading cause of death in the developed world [5].

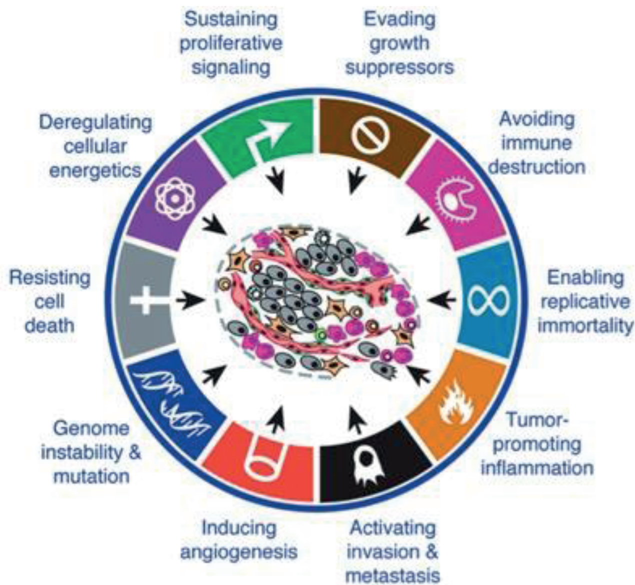

Figure 1. Hallmarks of cancer (modified and reprinted with the permission of Elsevier [3]).

4.2 Malignant melanoma

Malignant melanoma is a cancer form that arises from melanocytes, a cell type found in the basal layer of epidermis, hair bulbs, eyes, ears and meninges (membranes surrounding the central nervous system). The melanocytes comprise approximately 5-10% of all cells found in the basal layer of epidermis, and will through a process of melanogenesis produce melanin, the main pigment giving rise to the colour of our skin and hair. Melanin is transported to nearby keratinocytes, where it forms a cap on top of the nucleus of mitotically active cells and protects them from damaging effects of ultraviolet (UV) radiation [6].

4.2.1 Epidemiology and risk factors

The incidence rate of melanoma is steadily rising for both genders, and is thought to be attributable to both increased awareness, as well as augmented exposure to the sun [5,7].

Norway is among the European countries with the highest incidence rates, a rate which has increased nearly 10 times over the past 60 years (~2/100.000 (1953-1957) to ~19.6/100.000 (2008-2012)) [8].

Several risk factors have been found to increase the likelihood of developing melanoma.
Exposure to ultraviolet radiation (UV): Sunburns, primarily caused by UVB exposure (290-320nm), has been implicated as a potential contributing factor to the pathogenesis of melanoma. Animal studies have, however, demonstrated that UVA irradiation (320-400nm) can also trigger melanomagenesis [9,10]. The correlation between sun exposure and melanoma is supported by both epidemiologic evidence, as well as biological findings linking exposure to UV-radiation to occurrence of DNA damage [11,12].
Pigmentation: People with a fair complexion and red hair, who tan poorly, freckle and easily get sunburns, have increased risk of developing melanoma. In line with this, melanoma occurs more commonly in Caucasians, compared to people of other ethnic origins [13].
High number of melanocytic nevi: Presence of a high number of melanocytic nevi, dysplastic nevi and atypical mole syndrome (AMS) have been shown to augment the risk of developing melanoma [14].
Genetics: Two high-penetrance genes have been associated with hereditary melanoma; *CDKN2A* and *CDK4* [15]. A previous- or family history of melanoma has been found to increase the risk of developing melanoma, even though exact predisposing genes have not been identified in all cases [12]. Approximately 5-12% of all cutaneous melanomas are due to hereditary factors [16].
Age: The incidence of melanoma rises with increasing age [12].
Gender: While the incident rate is higher in women until the age of 40, melanoma in general is more common in men [12]. The sites of the body where melanoma arises have been shown to differ between the genders, and are thought to be related to different trends in carcinogenic exposures (UV-radiation) [17].

4.2.2 Melanoma stages

It is of great importance to identify a melanoma at an early stage in order to prevent it from metastasising. The ABCDE criteria are a much used tool to assist both health care providers and patients in evaluating potential melanomas. The mnemonic ABCDE considers different

aspects of the nevi; Asymmetry, Border irregularities, Colour variation, Diameter and Evolution (nevi that changes over time). Suspicious pigmented lesions should be biopsied [18].

The American Joint Committee on Cancer (AJCC) has recommended using the 'Tumour-Node-Metastases (TNM) grading system when dealing with melanomas. Tumours graded by this system are evaluated for thickness and ulceration (T), amount of metastatic lymph nodes (N) and sites of distant metastasis and level of serum Lactic Dehydrogenase (M) [19]. The Clark model and Breslow's depth are used to grade melanomas according to the TNM criteria. The Clark model depicts the stepwise transformation from melanocytes to melanoma [20] and Breslow's depth describes how deeply tumour cells have invaded the skin [21].

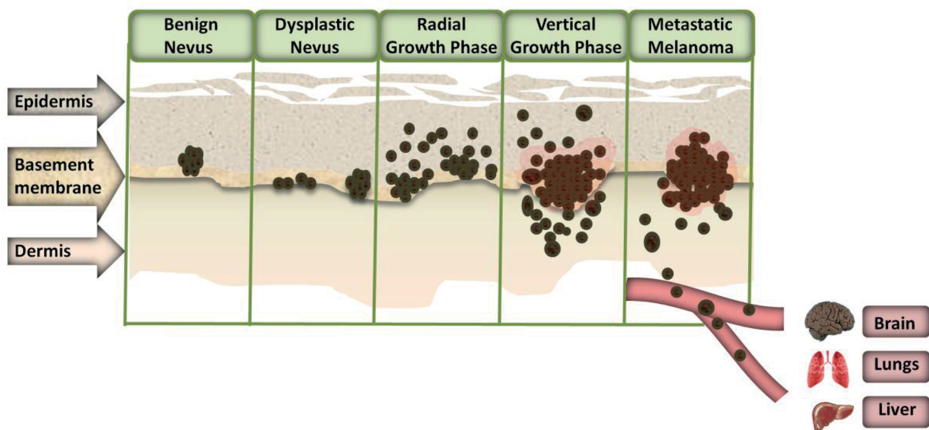

Figure 2. Progression of melanoma. The Clark model depicts the stepwise progression of a melanocyte to a mole and subsequently to melanoma. However, it has been shown that only 26% of melanomas arise from nevi, suggesting alternative pathways that can bypass the nevi as an intermediate step [22].

The staging system is used to sum up how far the cancer has spread, where the TNM values are combined to give an overall stage (0-VI). Patients with lower stages of melanoma have a better outlook for curative treatment or long-term survival.

4.2.3 Melanoma subtypes

Cutaneous melanoma (related to the skin) can based on clinical findings and pathologic growth patterns be classified into four subgroups; superficial spreading (70%), nodular (15%), acral lentiginous melanoma (10%) and lentigo maligna (5%) [23].
In addition to cutaneous melanoma, melanoma can also arise in the eye (uveal/ocular melanoma) and mucosal membranes of the body (mucosal melanoma). Current efforts are being made to more accurately classify melanoma according to their molecular profile; however these findings need to be further validated in larger cohorts [24,25].

4.2.4 Molecular alterations commonly found in melanoma

A large number of genes have been found to be mutated or altered in melanoma, these are often subtype-specific and some occur more frequently than others. The majority of the commonly found mutations affect two signalling pathways that regulate proliferation and survival, the Mitogen-activated protein kinase/Extracellular regulated signalling kinase (MAPK/ERK) and Phosphoinositide 3 kinase/V-Akt murine thymoma viral oncogene homolog (PI3K/Akt) pathways (Figure 3).

v-raf murine sarcoma viral oncogenes homolog B1 (B-RAF); is a central protein in the MAPK/ERK pathway, a signal transduction pathway involved in cellular proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis. Activating mutations in the B-RAF gene have been found in as much as 40-60% of cutaneous melanoma, most frequently in the V600E locus of the gene (80-90%) [26]. B-RAF mutations are also commonly found in nevi (80%), and are believed to be critical step in the initiation of melanoma tumourigenesis, although insufficient on its own [27].
Neuroblastoma RAS viral oncogenes homolog (N-RAS); is another protein involved in the MAPK/ERK- , but also in the PI3K/Akt signalling pathway. Activating mutations in the N-RAS gene have been found in approximately 20% of melanoma [28,29]. Mutations in B-RAF and N-RAS are almost always mutually exclusive [28].
c-KIT; is a receptor tyrosine kinase (RTK) necessary for melanocyte survival. Activating mutations of the encoding gene can lead to increased stimulation of a range of different signalling pathways, including MAPK/ERK- and PI3K/Akt [30]. In general, activating

mutations and/or gene amplifications of the c-KIT gene are relatively rare in melanomas, but can be found at a higher frequency in some of the smaller sub-groups (uveal (78%), mucosal (39%), acral (36%) melanoma, and melanoma arising in chronically sun-damaged skin (28%)) [31,32].

V-Akt murine thymoma viral oncogene homolog (Akt) and Phoshoinositide 3 kinase (PI3K); are among the key proteins involved in the PI3K/Akt pathway, a signal transduction pathway known to regulate a number of cellular processes, including growth, proliferation, apoptosis and cell migration [33]. Amplifications of the *Akt3* gene have been found in 25% of melanoma [34], while point mutations in the gene encoding PI3K have been reported in 2-6% [35].

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN); is a tumour suppressor gene that's protein is known to negatively regulate the PI3K/Akt pathway. Loss or reduction in PTEN protein expression has been shown in approximately 20% of melanoma [28].

Guanine nucleotide binding protein (G protein), q polypeptide (GNAQ) and guanine nucleotide binding protein (G protein), alpha 11 (GNA11); Activating mutations in either of these genes have been shown to increase MAPK/ERK signalling [36]. Studies have shown that 83% of all uveal melanomas harbour mutations in either of these genes [37,38]. Mutations in GNAQ and GNA11 are rare in cutaneous melanoma.

Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*); is a tumour suppressor gene that encodes two proteins involved in cell cycle inhibition, $p14^{ARF}$ (p14) and $p16^{INK4a}$ (p16). *CDKN2A* is the main gene involved in predisposition and melanoma pathogenesis [39]. Alterations to the *CDKN2A* locus (deletions and mutations) are found in approximately 20-50% of the cases of familial melanoma [40,41]. Somatic mutations and genetic alterations have also been observed in sporadic melanomas [42,43].

Cyclin Dependent Kinase 4 (CDK4); is a protein-serine kinase that is involved in the G_1 phase of the human cell cycle. Activating mutations [44] and allelic amplifications [45] in the *CDK4* gene, as well as over-expression of the protein [46], have been found in sporadic melanoma, leading to unrestricted kinase activity and aberrant cell cycle entry and uncontrolled cell proliferation [41]. Furthermore, *CDK4* is considered a melanoma susceptibility gene and germline mutations have been detected in 3 kindred worldwide [47]. The CDK4 pathway (p16-cyclin D-CDK4/6-retinoblastoma protein (RB1)) is associated with activating genomic alterations in more than 90% of cases of melanoma [43,48].

Microphthalmia-associated transcription factor (MITF); is a regulator of melanocyte differentiation and survival. Amplification of MITF has been observed in 10% of primary cutaneous- and 21% metastatic melanomas [49]. The exact role of MITF in melanoma has yet to be determined [30].

Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A); is a protein encoded by a gene that was newly discovered to be mutated in 33% of melanomas. The gene was identified after comprehensive exome sequencing and suggests involvement of glutamate signalling [50].

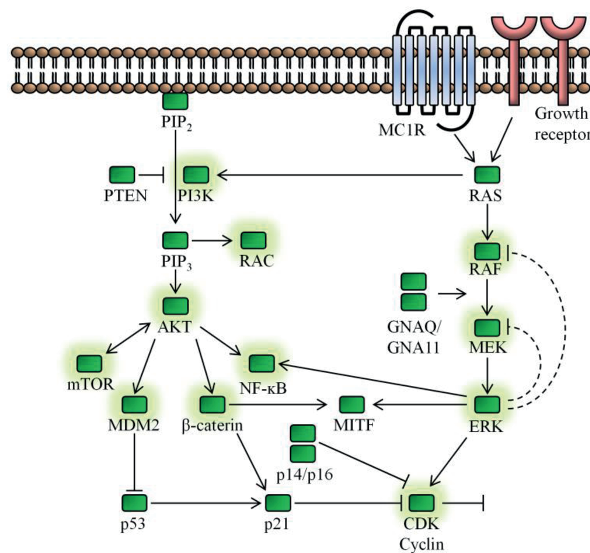

Figure 3. Common deregulated signalling pathways in melanoma. Squares with a green glow indicate proteins that are druggable targets. Adapted from [51].

4.2.5 Current treatment alternatives

Early-stage melanomas can, in most cases, effectively be treated by surgery alone. Additional treatment alternatives are, however, required for more advanced cancers.

For the treatment of patients with advanced disease, six drugs have been approved by the US Food and Drug Administration (FDA); Dacarbazine (1975), Ipilimumab (2011), Vemurafenib (2011), Dabrafenib (2013), Trametinib (2013) and Pembrolizumab (Keytruda) (2014) [52].

Each of the listed drugs exerts particular mechanisms of action, and is associated with drug specific side-effects.
Even though targeted treatment and immunotherapy have in many cases led to promising anti-tumour effect, development of resistance and adverse effects are a major problem [52].

Traditional chemotherapy

Dacarbazine has for years been first line treatment for melanomas. It has an unclear mechanism of action, but has been proposed to inhibit DNA synthesis and lead to alkylation of DNA bases, by so preventing tumour proliferation and growth [53]. Tumour responses have been seen in 10-15% of the patients; however these responses have not been reported to prolong median disease-free- or overall survival [54].

Targeted therapy

Vemurafenib and *Dabrafenib* are inhibitors of V600E mutant BRAF that limit the activity of the MAPK/ERK signalling pathway. Treatment with Vemurafenib has resulted in complete or partial tumour regression in the majority of melanoma patients carrying B-RAF V600E mutations, with a medium overall survival of 15.9 months [55,56]. Likewise, a median overall survival of 13.1 months has been reported in patients treated with Dabrafenib [57].
Trametinib is an inhibitor against MEK1 and MEK2 that has been approved as a single agent treatment of B-RAF V600E or V600K mutation-positive unresectable- or metastatic melanoma. A clinical study showed that melanoma patients (B-RAF^{mut}) treated with Trametinib had an improved overall survival (81% after 6 months), as compared to chemotherapy (Dacarbazine/Paclitaxel, 67% after 6 months) [58].
Clinical phase III trials have demonstrated increased median relapse-free survival of patients treated with a combination of Dabrafenib and Trametinib, as compared to single-agent treatment, and have led to FDA-approval of combined administration of the drugs (2014) [59]. Despite initial response, resistance to B-RAF and MEK inhibitors develops at a median time of 6 months after treatment initiation, and have been associated with reactivation of the MAPK signalling pathway, or activation of compensatory pathways such as the PI3K network [51,60,61] .

Immunotherapy

Ipilimumab is a monoclonal antibody that targets CTLA-4, thereby blocking the latter's ability to inhibit T-cell activation and proliferation. Treatment with Ipilimumab thus enhances the immune response against melanoma. In an earlier study, the median overall survival of patients treated with Ipilimumab was reported as 10.1months [62]. In another study, 4-year survival rates have been reported in as much as 37.7% to 49.5% of treatment-naïve patients receiving Ipilimumab [63].
Pembrolizumab is a monoclonal antibody against PD-1 receptors; the latter are negative regulators of T-cell effector mechanisms that limit immune responses against cancer. Treatment with Pembrolizumab has resulted in a high rate of sustained tumour regression in patients, including those previously treated with Ipilimumab [64].

4.2.6 Emerging therapeutic targets

Targeted therapy of melanoma is currently focused primarily on attenuating the MAPK/ERK- and PI3K/Akt signaling pathways, and improving the durability of the response by developing personalized combination strategies and better dosing schedules [65]. Several clinical trials are ongoing for patients with *B-RAF* mutations where B-RAF/MEK inhibitors are combined with each other or inhibitors of for instance Heath Shock Protein 90 (HSP-90), PI3K, Akt and CDK4/6 (ClinicalTrials.gov).
The therapeutic alternatives for patients with *N-RAS* mutations have so far been limited, since designing drugs that directly target N-RAS has been a challenge. Likewise, finding eligible strategies to treat melanomas that are *B-RAF/N-RAS* wild type have proven even more elusive [65]. Clinical trials are assessing the treatment potential of MEK inhibitors in these patient groups; however the responses appear to be sub-optimal. Aiming to improve the response, combined inhibition of MEK and PI3K, Akt, cyclin D/CDK4 and cyclin D3/CDK6, are currently being investigated (ClinicalTrials.gov).
The recent years advances in the field of immunotherapy have led to treatment alternatives that have improved survival and durability of response in patients [66]. Several clinical trials with immunotherapeutic agents are currently ongoing or underway. New immunotherapies are also being tested, both alone and in combination, including agents that are directed at T-cell

regulatory pathways which could enhance anti-tumor activities, such as lymphocyte activation gene 3 (LAG3) and T-cell membrane protein 3 (Tim3) [66].
Immunotherapeutic agents are also being investigated in combinations of targeted therapy, such as B-RAF inhibitors (ClinicalTrials.gov).

4.3 The cell cycle

The cell cycle is the orderly set of events leading to cell division, and consists of four distinct phases; Gap 1 (G_1), DNA Synthesis (S), Gap 2 (G_2) and Mitosis (M) (Figure 4). During the gap phases, the cell increases in size and ensures that everything is ready for the following phase. DNA replication occurs between the two gap phases and results in chromosome duplication. During the mitotic phase the cell physically divides itself in two. Under favourable conditions cells may commit to a new division by passing a point of no return in G_1 termed 'Restriction point'. Most cells in the adult, human body are non-dividing, and reside either in a resting state called G_0 (quiescence) where they remain dormant until stimulated to re-enter the cell cycle, or alternatively a permanent state (senescence) from which they are unable to return [67].

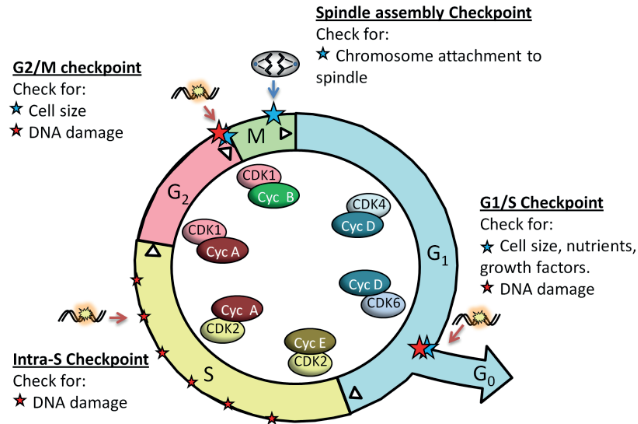

Figure 4. Overview of the cell cycle and its checkpoints. The cell monitors internal and external conditions, as indicated under the respective checkpoints, and may halt the cell cycle under unfavourable circumstances. The red stars signify DNA damage checkpoints, and the blue stars are checkpoints where the cell monitors external or internal conditions (as indicated in the figure). Overview of the cyclin/CDK complexes present in different stages of the cell cycle (inner circle).

Cell cycle Regulation

Progression through the different phases of the cell cycle is directional and tightly regulated. Transition to the next phase only occurs with the correct assembly and activation of cyclin and Cyclin Dependent Kinase (CDKs) complexes. The CDKs form the catalytic subunit of the complex, and are stably expressed throughout the cell cycle. Cyclins, on the other hand, are expressed in a phase-specific manner, and function as the regulatory subunit of the hetrodimer. The oscillating expressions of the different cyclins are achieved by their timely synthesis and ubiquitin-mediated proteolysis, resulting in phase specific cyclin/CDK combinations (Figure 4). In order to become fully active, the assembled cyclin/CDK complex needs to be phosphorylated by a CDK-Activating Kinase (CAK) [68].

In response to extracellular signals (such as growth factors) cyclin D is produced in the early stages of the G_1 phase [69]. Cyclin D binds to CDK4 and CDK6, forming active cyclin/CDK complexes that in turn phosphorylate the retinoblastoma susceptibility protein (Rb). Upon phosphorylation, Rb dissociates from its binding partner E2F, thereby activating the latter

transcription factor [70]. Activated E2F can then transcribe various genes encoding proteins that are necessary for the transition to S-phase; these include cyclin E, cyclin A and DNA polymerases [71]. Activation of the cyclin E/CDK2 complex leads the cell from G_1 to S-phase. Cyclin E is slowly degraded during S-phase, and its partner CDK2 now binds to cyclin A which allows the cell cycle to progress to late S phase. CDK1 is then activated by A-type cyclins at the later stages of S phase, which contributes to driving the cell towards mitosis [72]. At the onset of mitosis, CDK1 forms a complex with cyclin B which drives the cell through the final stages of the cell cycle.

CDK activity is also regulated by two families of CDK inhibitors (CDKIs): Inhibitor of kinase 4 (INK4) proteins ($p16^{INK4a}$ (p16), $p15^{INK4b}$ (p15), $p18^{INK4c}$ (p18) and $p19^{INK4d}$ (p19)) and the CDK interacting protein/kinase inhibitory protein (Cip/Kip) family ($p21^{Cip1/WAF}$ (p21), $p27^{Kip1}$ (p27) and $p57^{Kip2}$ (p27)). Additionally, activity of CDK1 and CDK2 may be restricted by inhibitory phosphorylation on the tyr15 and tyr14 residues mediated by Myt1 and Wee1. The latter inactivating phosphorylations can again be removed by the CDC25s.

Cell cycle checkpoints

Cell cycle checkpoints are regulatory pathways that control the order and timing of transitions and may stop cell cycle progression under unfavourable conditions. These signal transduction pathways may respond to both extrinsic and intrinsic factors, and malfunctions of such checkpoints have been implicated in tumourigenesis [73]. Four major checkpoints are found; G_1/S, Intra-S, G_2/M and the spindle assembly checkpoint (Figure 4).

4.4 Cell death

Under certain circumstances and upon receiving appropriate signals, a cell can activate a controlled process of self-destruction. Based on morphological appearance, this process of cell death can be defined as apoptotic, necrotic, autophagic or associated with mitosis [74]. The apoptotic cell death is highly controlled and has been linked to morphological alterations such as cell shrinkage, membrane blebbing, chromatin condensation, decreased cytoplasm and formation of apoptotic bodies [75]. Morphological alterations usually found in necrotic cells,

on the other hand, include cytoplasmic swelling, rupture of the plasma membrane and chromatin condensation [74]. This type of cell death can be either controlled or random, and leads to intracellular content being spilled into the nearby tissue which potentially causes an inflammatory response. Autophagic cell death is a process where proteins and organelles are degraded by lysosomal proteases. This type of cell death occurs without chromatin condensation, and is associated with formation cytoplasmic vacuoles (double-membrane subcellular compartments containing degenerating organelles or cytosol)[76]. Mitotic catastrophe is associated with aberrant mitosis, and is morphologically distinct from other forms of cell death by the presence of micronuclei (containing chromosomes or chromosome fragments) and presence of two or more nuclei that can be of different size.

Apoptosis

Apoptosis is a highly regulated form of cell death, which can be initiated by intracellular stress signals and/or extracellular ligands (Figure 5).
Intracellular stress signals include withdrawal of growth factors, DNA damage, loss of cell anchorage, oxidative stress or oncogene activation, and may trigger the 'intrinsic pathway of apoptosis'. This pathway initiates apoptosis through mitochondrial outer membrane permeabilization (MOMP), a process that is carefully regulated by the Bcl-2 family. The Bcl-2 proteins consist of both pro- (e.g. Bim, Bad, Bid) and anti-apoptotic members (Bcl-2, Bcl-xl, Mcl-1), and a shift in their balance may lead the cell towards apoptosis [77]. It has been proposed that pro-apoptotic Bcl-2 proteins then activate Bak and Bax, which after several conformational changes and oligomerization can form pores in the mitochondria [78]. The following drop in mitochondrial membrane potential leads to the release of pro-apoptotic proteins such as cytochrome c, SMAC/DIABLO, AIF and EndoG. Upon its cytoplasmic release, cytochrome c forms a complex (Apoptosome) with Apaf-1 and pro-caspase 9, and this leads to an activating cleavage of the latter protein [79]. Caspase 9 is an initiator caspase that can activate effector caspases (such as caspase 3) that are important in executing apoptotic changes in the cell which includes cleavage of DFF40/45 (leading to DNA fragmentation [80]) and –PARP-1 (leading to its inactivation, and thus inhibiting its functions in DNA repair that given the circumstances could otherwise drain the cell of ATP [81]). The cytoplasmic release of SMAC/DIABLO, on the other hand, leads to inhibition of IAPs (Survivin, Livin and XIAP) which are anti-apoptotic proteins that can bind to effector

caspases and thereby prevent their actions [82]. Following their mitochondrial release, AIF and EndoG are translocated to the nucleus where they exert their apoptotic function. The latter proteins have also been implicated in caspase independent apoptosis [83].

The extrinsic pathway is activated through binding of ligands (such as TRAIL, Fas and TNFα) to death receptors on the cell surface. This leads to clustering of death receptors and intracellular recruitment of adaptor proteins (e.g. FADD and TRADD) and pro-caspase 8 or 10, thereby giving rise to the Death Inducing Signaling Complex (DISC) and subsequent activation of the latter caspases [84]. Caspase 8/10 can then activate effector caspase 3, as well as the intrinsic pathway though cleavage of Bid [85].

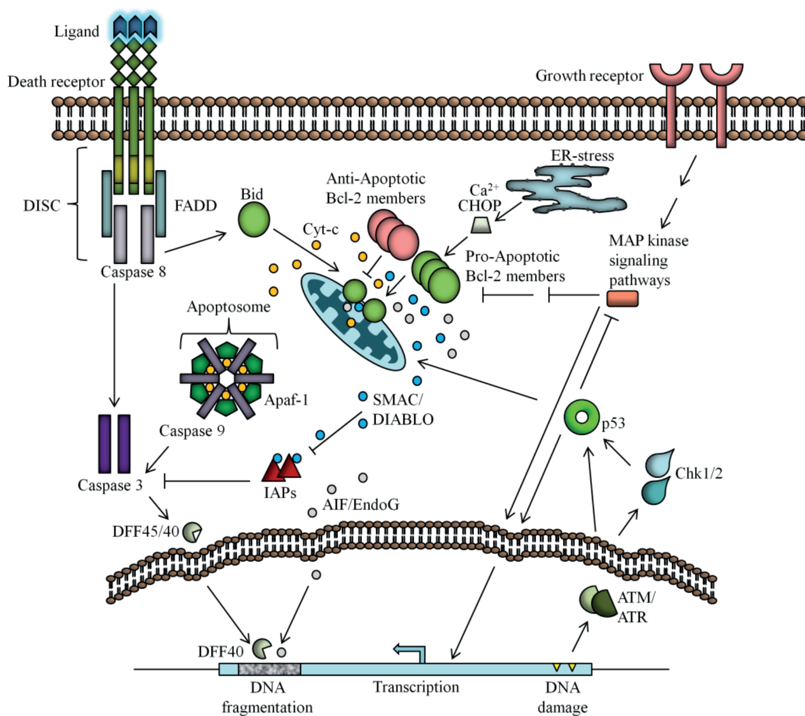

Figure 5. Simplified overview of apoptosis. Initiation of apoptosis can occur through the binding of an extracellular ligand to death receptors (extrinsic pathway), or through mitochondrial permeabilization (intrinsic pathway) as a response to DNA damage or intracellular stress, and lead to activation of initiator- and executioner caspases.

4.5 DNA damage response

DNA damage occurs both as a consequence of stress from endogenous (for instance reactive oxygen species which arise from normal cellular metabolism) - and exogenous sources (such as UV light or chemicals in the environment). Over the course of time, the cell has developed mechanisms to guard itself against potentially harmful DNA damage that can lead to mutations that facilitates tumourigenesis. These systems, collectively called DNA damage response (DDR), are designed to prevent DNA damage from being passed on in dividing cells, and include cell cycle regulation, DNA repair and apoptosis [56].

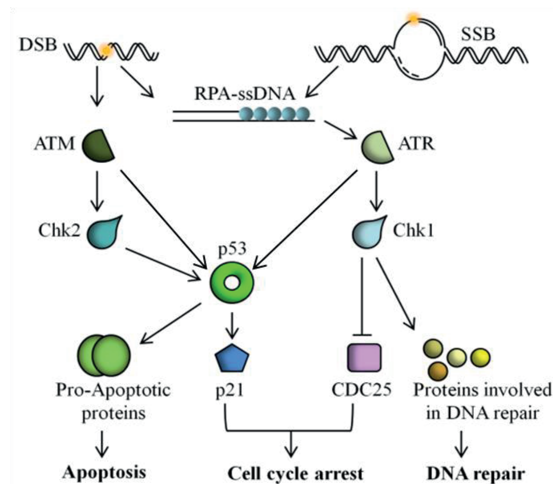

Figure 6. Activation of DNA damage response pathways (adapted from [86]).

In response to DNA double-stranded breaks (DSB) ATM is activated and phosphorylates p53, directly or indirectly through Chk2. This leads to accumulation of p53 and subsequent activation of its downstream target genes. Transcriptional activation of genes encoding proteins, such as Bax and Puma, promotes apoptosis, whilst transcription of p21 leads to cell cycle arrest. Following the occurrence of Single-Stranded breaks (SSB), or resection of DSB, single-stranded DNA (ssDNA) is coated with Replication Protein A (RPA) which leads to recruitment and activation of ATR. The latter can then phosphorylate many intracellular substrates including Chk1 and p53. Activated Chk1 promotes cell cycle arrest through adding an inhibitory phosphorylation on CDC25, and/or DNA repair by activating proteins such as RAD51, FAND2 and FANCE.

4.5.1 DNA damage checkpoints

There are three main DNA damage checkpoints in the cell cycle; G_1/S, intra-S and G_2/M (Figure 4 and 7). The cell may arrest in the G_1/S and G_2/M checkpoints in response to DNA damage, whilst progression is slowed down rather than stopped in S-phase.

The G_1/S DNA damage checkpoint is primarily activated through the ATM-Chk2-p53-p21 and ATM-Chk2-CDC25a pathways [87]. The full activation of the first of these pathways involves transcriptional activity which leads to increased expression of proteins such as p21, a CKI that is critical for inhibiting S-phase entry, and causes a potentially permanent cell cycle arrest [88]. The ATM-Chk2-CDC25a signalling pathway, on the other hand, only requires posttranslational modifications, which makes its activation more rapid and transient [89]. Chk2 mediated phosphorylation of CDC25a inhibits its activity, marks it for degradation and/or promotes its sub-cellular relocation to the cytoplasm [90]. This limits the ability of CDC25a to remove the inhibitory phosphorylations (tyr14 and tyr15) on CDK2 in late G_1 phase [91]. The CDK2 activity is further decreased by Wee1, the complimentary counterpart of CDC25a, which instead adds inhibitory phosphorylation on CDK2 [92,93]. Although ATR-Chk1-CDC25a signalling has been reported in late G_1, the checkpoint is thought to be mainly mediated by the described ATM-dependent signalling pathway [94,95].

The intra-S DNA damage checkpoint is manifested by decreased DNA synthesis rather than a full arrest, and can thus be described as a transient phenomenon [96]. The intra-S phase checkpoint response is activated by the ATM-Chk2-CDC25a and ATR-Chk1-CDC25a signalling pathways that lead to reduced activity of CDK2 [95]. Checkpoint mechanisms are triggered by the formation of unusually long stretches of ssDNA that occur at stalled/collapsed replication forks, SSB or during resection of DSB. These regions of ssDNA attracts RPA, which coats the strand and acts as a landing pad for the recruitment and activation of checkpoint signalling proteins, such as ATR [97,98]. The latter kinase then activates Chk1, which in a similar manner to Chk2 can inhibit CDC25.

The G_2/M DNA damage checkpoint stops cells that have escaped previous arrests, as well as those with DNA damage that has occurred in G_2 phase. The G_2/M cell cycle delay/arrest is usually a result of acute mechanisms that operates through post-translational alterations of effector proteins; ATR/ATM activates Chk1/Chk2 that subsequently inhibits the CDC25

phosphatases; CDC25a, b and c (all of which have been implicated in G_2/M checkpoint regulation) [99]. It has been proposed that these rapid mechanisms that target CDC25 produce a longer cell cycle delay in G_2, than in previous phases, in particular when ATR-Chk1 signalling is involved [100]. A p53-dependent signalling pathway, involving transcription of p21, has been described; however its exact role in regulating the G_2/M checkpoint remains unclear [89].

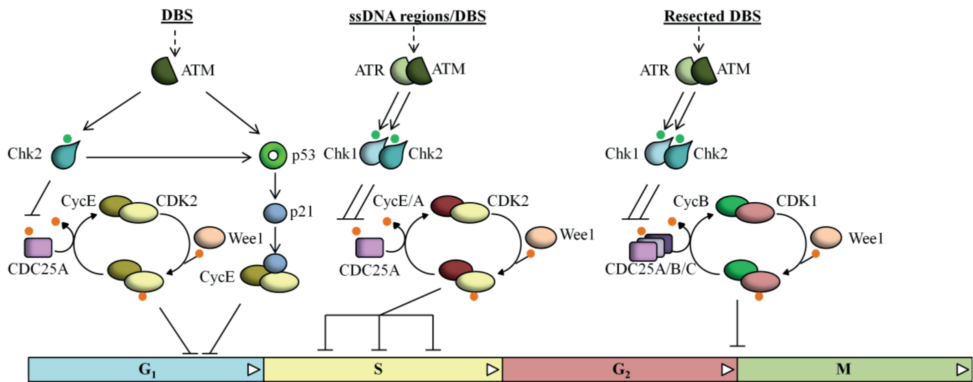

Figure 7. A simplified overview of mammalian signal transduction pathways involved in cell cycle arrest following Ionizing Radiation (IR). The figure is based/modified from [101]. Following DSB in G_1, two ATM-dependent checkpoint pathways are activated and lead to inhibition of CDK activity; ATM-p53-p21 that requires transcriptional activity, and ATM-Chk2-CDC25 that causes a transient arrest through post-translational modifications. In response to DNA damage in S-phase, the ATM/ATR-Chk2/Chk1-CDC25 signalling pathways are activated and lead to a delay in cell cycle progression, rather than a full arrest. DNA damage detected in the G_2/M checkpoint causes an immediate arrest through targeting of CDC25a, b and c. A p53-dependent arrest has been described in G_2/M, and possibly works as a back-up to the transient mechanisms.

4.5.2 DNA repair

To combat threats posed by DNA damage, the cell needs to detect DNA lesions, signal their presence and promote their repair. The cell has evolved multiple distinct DNA repair

mechanisms to deal with the variety of DNA lesion that arise (reviewed in [102]). In response to DSB, possibly the deadliest form of DNA lesions, mammalian cells have two main pathways for repair: Non homologous End Joining (NHEJ) and Homologous Recombination (HR).

HR is an error-free repair mechanism where a homologous chromatid serves as a template to guide repair of the broken strand [103]. Repair by this pathway can thus only occur during S- and G_2 phases when a sister chromatid is available.
NHEJ, on the other hand, can take place in all phases of the cell cycle, and is the major pathway for repairing DSBs. The repair pathway is prone for errors since little or no sequence homology is used when joining the DNA ends. This makes it "quick and dirty", as it is associated with loss of nucleotides and chromosomal translocations or fusions [104].
HR is the preferred repair pathway during S-phase, where it has a central role in repairing DSBs that arise at collapsed replications forks. NHEJ dominates repair in G_1 and G_2 phases, although both repair mechanisms are available in the latter cell cycle phase [105].
In cases where DNA damage is beyond repair, checkpoint mechanisms will eliminate such potentially hazardous cells either by initiating permanent cell cycle arrest or cell death.

4.5.3 DNA damage and apoptosis

In response to DNA damage that is too severe to repair, the cell can initiate apoptosis through a process that usually involves the p53 network (Figure 5). ATR/ATM are activated following DNA damage and can phosphorylate p53, thereby increasing its activity [106]. Further phosphorylation mediated by Chk1/Chk2 leads to p53 stabilization [107,108]. Activated p53 can initiate apoptosis though several mechanisms; transcription of pro-apoptotic proteins (e.g. Apaf-1 [109], Puma [110], Noxa [111], Bax [112] and Bid [113]), transrepression of genes encoding anti-apoptotic proteins (e.g. Bcl2 [112] and Survivin [114]) and through direct interaction with pro- and anti-apoptotic Bcl-2 proteins that results in MOMP [115,116]. Moreover, activated p53 can counteract survival signals from the microenvironment, for instance through regulating PTEN, which is a negative regulator of the PI3 kinase signaling pathway [117]. Whereas low levels of DSB have been proposed to cause a minor increase in p53 leading to cell cycle arrest, high levels of DSB is believed to elevate the levels of p53 over a certain threshold and drive the cell towards apoptosis [118].

4.5.4 DNA damage response and cancer

DDR is central to cancer in many ways; dysfunctions in these systems are associated with predisposition to cancer, as well as onset of carcinogenesis since most carcinogens are genotoxic. Furthermore, failure of DDR mechanisms facilitates the tumourigenic evolution, as this is driven by mutations and chromosomal instability. The DDR system is also relevant for the effectiveness of genotoxic cancer therapy that targets proliferative cells. Finally, genetic and epigenetic alterations that prevent cell death (e.g. apoptosis) are also central to the development of resistance to therapy.

DNA damage response and malignant melanoma

Genetic instability, one of the hallmarks of cancer, is closely linked to abnormalities in the DDR machinery. There are several classic examples of how defects in specific DNA repair mechanisms can predispose individuals to cancer. For instance, individuals with the *Xeroderma pigmentosum* (XP) syndrome have alterations in genes involved in DNA excision repair, which makes them especially sensitive to UV-induced DNA damage. Persons with XP have a 10.000-fold increased chance of developing skin cancer, including malignant melanoma [119].

Aberrations in G_1/S checkpoint components, such as p53, have been found in a vast number of human cancers [120]. Although mutational inactivation of p53 is relatively rare in melanoma, its function is often abnormal as reflected in its failure to induce cell cycle arrest and apoptosis [121-123]. As previously discussed, alterations in the *CDKN2A* gene, encoding p14 and p16, are commonly found in melanoma. Whereas mutational inactivation of p14 leads to proteosomal degradation of p53 and subsequent accumulation of DNA damage, mutations in the p16 protein, on the other hand, renders the cell with impaired capacity to inhibit CDK4 and consequently allows unchecked cell cycle progression in G_1 phase [124].

Studies of melanoma gene expression and disease progression have identified 254 genes with prognostic implications for metastasis-free survival of patients with primary disease. Many of the genes were associated with DDR, and in particular repair of DNA double-strand breaks [125,126]. Interestingly, over-expression of specific DNA repair genes (e.g. CHEK1 and BRCA1) was associated with a poor prognosis. The current understanding of this

phenomenon is that while genetic instability is necessary for the cell to undergo malignant transformation, some genetic stabilization is required for the primary tumour cell to be stable enough to give rise to distant metastasis [127].

4.6 Characterization of potential targets and treatment

4.6.1 The synthetic retinoid, CD437

Retinoids are a group of compounds that consists of natural and synthetic vitamin A derivates and analogues that are implicated in a broad range of biological processes including apoptosis, growth and homeostasis [128]. Some of the natural retinoids have shown great potential as treatment for cancer, such as ‘All-Trans Retinoic Acid’, which is currently included in the treatment of patients with Acute Promyelocytic leukemia (>90% remission)[129]. Since natural retinoids have had limited effect in other cancer forms, as well as caused adverse effects in patients, synthetic analogues have been developed [130]. While the effect of natural retinoids are believed to be mediated mainly through their binding to nuclear receptors (retinoic acid (RAR) - and retinoid X receptors (RXR)), the mechanisms of action of the synthetic retinoids are less clear.

6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid (CD437/AHPN) is a synthetically developed retinoid (RARγ agonist) that has been reported to induce apoptosis and cell cycle arrest in a broad range of cancer cell lines, including melanoma [131,132]. The underlying mechanisms appear as cell line dependent and involve a range of different cellular organelles, such as the endoplasmatic reticulum [133], lysosomes [134], mitochondria [135] and nucleus [136]. For instance, in a previous study, CD437 was shown to induce apoptosis in a nucleus-independent manner, thereby indicating that transcriptional activities are not a prerequisite for the effect in these cells [135]. In line with this, RAR-independent induction of apoptosis by the retinoid has been found in some studies, while a partial dependence on such receptors has, on the other hand, been reported in other cell systems [132,136,137]. Cell death following treatment with CD437 may occur in a caspase dependent- or independent manner, depending on the cell line [138,139]. Moreover, genotoxic stress including double-strand breaks in DNA has been found in treated cells, leading to activation of p53 and apoptosis [140].

4.6.2 TRAIL

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a cytokine of the TNF superfamily that can induce apoptosis in a tumour-selective manner through its binding to DR4 and DR5 death receptors on the cell surface, by so activating the extrinsic pathway of apoptosis [141,142]. Additionally, TRAIL can bind to two decoy receptors (DcR1 and DcR2) in human cells with the same affinity as to the death receptors, however binding to the decoy receptors do not initiate apoptosis [143,144]. A difference in the composition of decoy-and death receptors on the cell's surface between normal and malignant cells was initially proposed as a possible rationale behind the tumour-selective apoptosis induced by TRAIL; however this hypothesis has been questioned by newer studies [145].
Recombinant versions- and agonistic antibodies of human TRAIL have been developed for the purpose of cancer-therapy, such as Lexatumumab, an agonistic TRAIL receptor-2 (DR5) monoclonal antibody. Despite showing promising results in preclinical studies, TRAIL signaling does not always result in apoptosis in cancer cells [146]. The mechanisms underlying resistance to TRAIL treatment include induction of pro-survival responses, such as increased anti-apoptotic Bcl-2 proteins, IAPs, MAPK and Akt, as well as absence or low expression of death receptors [147,148]. Combinational therapy represents a potential strategy for treating TRAIL resistant cancers. In line with this, Lexatumumab has shown promising results in preclinical studies of melanoma, in particular in combination with other agents such as Dacarbazine [149] and Anisomycin [150]. Treatment with Lexatumumab is generally well tolerated as assessed in a Clinical phase I trial of pediatric patients with solid tumours [151].

4.6.3 Wee1

Wee1-like kinase is an evolutionarily conserved Ser/Thr and Tyr kinase which is prominently active during the S- and G_2 phases of the cell cycle [93,152,153]. Its activity is regulated through increased synthesis during S-and G_2 phases, and by inhibitory phosphorylation and degradation during mitosis [93]. Wee1 was first described in fission yeast (Schizosaccharomyces pombe) and given its name based on the observation that yeast lacking the protein had a smaller phenotype [153]. The abnormal size of yeast cells lacking Wee1 is related to the consequent dysfunction of the G_2/M checkpoint, leading to premature mitotic entry. Wee1 negatively regulates cell cycle progression by adding inhibitory phosphorylations (tyr15) on CDK1 and CDK2, thereby limiting the activity of the latter kinases [92,93]. The

inhibitory phosphorylations of CDKs permit the completion of DNA replication in S-phase and a timely onset of mitosis [154]. In single cell organisms, such as yeast, Wee1 depletion can be endured, however its absence is associated with embryonic lethality in mice [155]. Besides its function in halting the cell cycle in response to DNA damage, Wee1 plays an important role in the unperturbed cell division [156]. During S-phase, replication origins are fired at an organised and timely manner to duplicate DNA, and this process is dependent on a balanced level of CDK2 activity. Depletion of Wee1 has been shown to increase CDK2 activity and firing of replication origins, which causes a subsequent nucleotide shortage in the cell. This presumably leads to reduced replication fork speed and ultimately DSB [157]. Besides its role in controlling CDK activity, Wee1 has been proposed to play a direct role in regulating Mus81-Eme1 endonuclease activity in S-phase, possibly by phosphorylation [158]. The Mus81-Eme1 enzyme complex is involved in replication recovery and initiation of homologous repair [159].

Additionally, Wee1 was recently suggested as an epigenetic modifier that can phosphorylate histone H2B (tyr37) in the nucleosomes and thereby suppress transcription of histones in late-S phase which is necessary in order to achieve the correct histone-DNA stoichiometry prior to mitotic entry [160].

Wee1 as a potential anti-cancer target

Genetic instability and increased proliferative index make cancer cells more sensitive to DNA damage than normal cells, a trait which has been exploited in cancer treatment for many years [3]. Despite initial responses to genotoxic agents, cancer cells often become resistant to therapy as they acquire the ability to repair DNA damage and survive. Simultaneous targeting of proteins involved in cell cycle regulation and DNA damage response, such as Wee1, has therefore been proposed as a possible way to enhance the effect of DNA-damaging agents. In line with this hypothesis, Wee1 inhibition has shown promising results in preclinical studies in combination with DNA damaging agents such as radiation, gemcitabine, cisplatin and 5-FU [158-161]. Preclinical studies have also reported an increased anti-tumor effect from combining Wee1 with a broader range of anticancer agents, for instance Heath Shock Protein 90 and Chk1 inhibitors [162,163]. Interestingly, targeting Wee1 alone has been shown to reduce proliferation [164,165] and induce apoptosis [166,167] in several cancer cell lines.

4.6.4 Chk1 and Chk2

Checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) are serine/threonine-specific proteins encoded by the CHEK1/CHEK2 genes, respectively, in humans. The proteins are both structurally and functionally distinct, and are activated through different mechanisms. Chk1 is activated by ATR in response to formation of lesions of ssDNA, and has essential functions in cell cycle regulation and DNA damage response (Figure 6 and 7, and reviewed in [161]). During S-phase, Chk1 plays an important role in safeguarding genome integrity by stabilizing stalled replication forks [162], controlling replication origin firing and -replication fork progression [163], and homologous recombination [164,165]. Furthermore, Chk1 also functions in the G_2/M transition where it controls mitotic entry in the unperturbed cell cycle [166], and stops progression in response to DNA damage [167]. Whereas Chk1 is primarily activated in response to DNA SSB, Chk2 on the other hand, is activated mainly in response to DSB by ATM. Some crosstalk between ATM and Chk1 has, however, been reported (reviewed in [168]). The cardinal role of Chk2 is to activate p53 in response to DNA damage in G_1/S, and thereby initiate cell cycle arrest or apoptosis [169].

Chk1/2 as potential anti-cancer targets

Based on its well-known role in the G2/M checkpoint, small-molecular inhibitors of Chk1 were originally developed as chemosensitizing agent for DNA-damaging drugs in p53 mutated cancer cells [177]. Due to dysfunctional G1/S checkpoint in the majority of cancers, these cells are more reliant on the G2/M checkpoint to repair DNA damage, a feature that should allow for selective targeting of cancer cell while sparing normal cells. The p53 mutation status is, however, not always a predicator of tumour sensitivity to the combination of Chk1 inhibitors and DNA-damaging agents [178]. Recent studies have emphasized the role of Chk1 in safeguarding the genome during replication as the mechanistic basis for the success of Chk1 (Chk1/Chk2) inhibitors [179]. Combination studies with Chk1 (or Chk1/Chk2) inhibitors and cisplatin have shown promising results in some reports, however little or no sensitization of cells was observed in other preclinical studies [178,180-182]. In the latter years, several Chk1 and Chk1/Chk2 inhibitors have been developed, not only as chemopotensiators, but also as single-agent therapies [183].

5. SUMMARY OF PAPERS

Paper I

Synthetic retinoid CD437 induces apoptosis and acts synergistically with TRAIL receptor-2 agonist in malignant melanoma.

Retinoids is a family of molecules consisting of natural- and synthetic derivatives of vitamin A that has been shown to have growth- and differentiating suppressive effects in cells. In this study, we investigated the anti-tumour effect of a synthetic retinoid, CD437, alone and in combination with a TRAIL death receptor-2 agonist, Lexatumumab, in two melanoma cell lines (FEMX-1 and WM239). Exposure to CD437 led to decreased viability, cell cycle arrest and induction of apoptosis. The particular response, however, varied between the two cell lines in terms of caspase-dependence and DNA-fragmentation. Interestingly, an increase of death receptor DR5 expression was observed following retinoid treatment. This observation spurred us to investigate if the anti-tumour effect of CD437 could be amplified by combined administration of Lexatumumab. In agreement with the hypothesis, combined treatment led to a synergistic decrease in viability in both cell lines.

Paper II

High expression of Wee1 is associated with poor disease-free survival in malignant melanoma: potential for targeted therapy.

Wee1 is a kinase that has a major cell cycle regulatory role in S- and G_2 phases through controlling CDK activity. The kinase has been implicated in regulation of replication initiation in the unperturbed cell cycle, in addition to halting cell cycle progression in response to DNA damage in G_2/M phase. In this paper we investigated the protein expression of Wee1 in a panel of patient-derived tissues of benign nevi and primary- and metastic melanoma, and found the level to increase in the direction of malignancy. When examining Wee1 expression in relation to previously described biomarkers involved in cell cycle regulation, we observed that a high level of the kinase was positively correlated with expression of p53, p21, Ki67, cyclin D3 and cyclin A. Likewise, high expression of Wee1 also correlated with thicker tumour, presence of ulceration and shorter relapse-free survival. These findings suggest that high expression of Wee1 in primary melanomas is an unfavorable prognostic indicator.

To investigate Wee1 as potential target for therapy, we depleted the kinase protein expression through siRNA mediated knockdown in three metastatic melanoma cell lines (WM239, WM45.1 and LOX). In two out of three cell lines, removal of Wee1 led to DNA damage, cell cycle arrest and apoptosis. Surprisingly, the inhibitory phoshorylation on CDK1, mediated by Wee1, did not decrease following kinase depletion in the unresponsive cell line (LOX), which may explain the divergent overall effect in this cell line. Together our results demonstrate an advantagous anti-tumour effect of targeting Wee1 in metastatic melanoma.

Paper III

Combined inhibition of the cell cycle related proteins Wee1 and Chk1/2 induces synergistic anti-cancer effect in melanoma.

Our previous study (paper II) implicated Wee1 as a potential target for therapy in melanoma. To further explore this concept, we investigated if the anti-tumour effect of targeting Wee1, by the promising MK1775 inhibitor that is currently undergoing clinical phase II trials, could be amplified by co-treatment with a Chk1/2 kinase inhibitor (AZD7762). Chk1 and Chk2 are key regulators of DNA damage surveillance pathways, and critical components of cell cycle checkpoint mechanisms. Whereas both inhibitors reduced cell viability as mono-agents, dual-administration resulted in a synergistic effect in our panel of metastatic melanoma cell lines. Likewise, combined targeting had an increased anti-tumour effect in spheroids and xenograft models, compared to single agent treatment. The observed anti-tumour effect following co-treatment was accompanied by premature mitotic entry, accumulation of DNA damage and apoptosis. These data demonstrate an eligible effect of simultaneous targeting of cell cycle regulatory proteins Wee1 and Chk1/2, and warrants for further investigation of such a treatment regimen.

6. METHOLOGICAL CONSIDERATIONS

6.1 Cell cultures and xenografts as models for testing anti-cancer drugs

In all three papers, metastatic melanoma cell lines have been used as model systems for determining the response to siRNA mediated knockdown of proteins and/or anti-cancer drugs. Additionally, primary cultures of fibroblasts and melanocytes were included in paper III. Cell cultures are an invaluable tool for determining efficacy and mechanism of action of novel drugs. The use of tumour cell lines allows researchers to test compounds under highly controlled and reproducible conditions. Among the advantages is the unlimited access to material (protein, RNA and DNA), worldwide availability of cell material and the opportunity for long-term storage of viable cells in liquid nitrogen. *In vitro* systems will, however, poorly reflect tumour heterogeneity, and cells grown *ex vivo* will lose some of the characteristics of the original cells over time. In line with this, a difference in protein expression, growth rate and response to stimuli have been observed when comparing cells from low and high passage numbers [170]. To minimize these effects, cells are thawed regularly and experiments performed on cells at low passage numbers.

Some of the complexity of cell-cell interaction is lost when cells are grown in monolayer cultures, and multicellular tumour spheroids have in some studies been shown as better models for drug screening [171,172]. In paper III, melanoma cell lines were grown in 3D cultures to test drug efficacy. Whereas WM983B cells appeared as tightly packed spheroids, WM239 and WM45.1 cells formed looser structures. In this regard, the lack of density in the latter cell lines could reflect less cell-cell interaction, absence of a pathophysiological gradient (e.g. oxygen, nutrients) and lack of true spherical geometry [173]. Although the spatial organization of spheroids is closer to the physiological conditions of a tumour than monolayer cultures, neither model can recapitulate the complexity of tumours and their interaction with the host. *In vitro* testing of drugs also fail to consider issues with drug delivery to affected tissues and toxicity towards essential parts of the organism, and these aspects are better evaluated by the use of animal models.

Xenograft models of melanoma, as used in paper III, can be established by injecting immunodeficient mice with tumour cells. Although, nude mice (foxn1^{nu}) lack thymus, T cell

deficiency is not complete and increased NK cell activity are seen in these mice. Consequently, the success rate is relatively low and only 20-40% of tumour cell lines are able to grow in nude mice [174]. This causes an extra level of selection of tumour cells, in addition to what is already posed by long-term culturing of cells. Xenotransplanted mice are relatively complex models for drug testing, however normal interaction between tumour cells and the microenvironment is not fully recapitulated and the lack of a functional immune system in the nude mice make the model deviate from actual tumour conditions. Unfortunately, it is generally a poor correlation between results from xenografts and human clinical studies [175]. Although there are no optimal models for testing novel anti-cancer drugs, the combined use of cell cultures and mouse models is likely the best option to predict the response.
In paper II and III, a cell line called 'WM45.1' has been included in many of the experiments. This cell line was established at the Wistar Institute in Philadelphia, and originally given the name '451-Lu' [176]. Several publications on melanoma cell lines named WM451 and WM45.1 can be found on 'pubmed.gov', and likewise with the names 451Lu and 451-Lu. We have performed cell line authentication to confirm that the WM45.1 cells used in our experiments are the same as the 451-Lu cell line. The mix-up of names has most likely occurred due to the many Wistar cell lines starting with the abbreviation WM (Wistar Memorial). In accordance with paper II and III, the cell line is referred to WM45.1 in this thesis.

6.2 Tissue material

The patient material used in paper II was selected from a melanoma archive that consists of paraffin-embedded biopsies from patients that have undergone surgery at either the Norwegian Radium- or regional hospitals since the 1980s. The archive consists of benign nevi and primary- and metastatic melanoma samples. Research on this material has been approved by the Regional Committee for Medical Research Ethics South of Norway (S-06151), and the Social and Health Directorate (06/2733).

6.3 SiRNA Transfection and inhibitors

In all three papers, small interfering RNA (siRNA) transfection and/or inhibitors have been used to down-regulate the expression or inactivate desired proteins in order to study the anti-tumour effect in their absence. SiRNA transfection is a method to transiently down-regulate the expression of desired proteins by using short dsRNA molecules (20-24bp) that bind to complementary mRNA and causes their degradation prior to translation [177]. One of the major challenges with this method is that the observed effects could be caused by non-specific knockdown of other genes with similarities in nucleotide sequence to the desired gene. A match of 11-15 nucleotides is sufficient to induce an off-target effect [178]. The use of low concentrations of siRNA can minimize the risk of off-target effects. In each experiment negative controls were included and contained the same nucleotide composition as the siRNA, but without substantial sequence homology to the genome (scrambled siRNA/siCtr). The siRNA oligonucleotides (Stealth RNAi) used in paper II and III were purchased from Invitrogen, and protein knockdown was verified by the use of Western blot analysis.

Several inhibitors were used in the articles, targeting; pan-caspases (Z-VAD-fmk, paper I), Wee1 (MK1775, paper III) and Chk1/2 (AZD7762, paper III). The use of inhibitors is a quick and efficient way for abrogating the function of a desired protein. Target specificity can, however, be a problem, especially when using high drug concentration. MK1775 is a highly selective inhibitor which has been shown to reduce Wee1 activity with a half-maximal inhibitory concentration (IC_{50}) of 5nM [179,180]. The inhibitor has shown affinity for 8 out of 224 tested kinases besides Wee1, of which YES1 was the only high affinity target [181]. AZD7762 is a potent and selective inhibitor of Chk1 (IC_{50} of 5nM) and Chk2 (IC_{50} of >10nM). The inhibitor has good enzyme selectivity (>10 fold) against more than a hundred other tested kinases, including CDK1, p38 and MAPKAP2 [86].

6.4 Measurements of viability

Viability assays were performed in all three papers, either by 3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), CellTiterGlo (CTG) assays and/or by cell count of trypan blue-excluding cells.

MTS is a colorimetric method for determining the amount of viable cells by measuring their ability to reduce MTS reagent, in the presence of phenazine ethosulfate, into a water-soluble formazan product [182]. The colored end-product can be measured by a spectrophotometer, and has a linear correlation with dehydrogenase activity in metabolically active cells.

CTG is a luminescence method for measuring viable cells by quantifying ATP. The CTG reagent contains detergents to rupture cells thereby causing the release of intracellular ATP, and ATPase inhibitors to stabilize it. The reagent also contains luciferin, Mg^{2+} and mutated luciferase that can generate a measurable luminescent signal. In addition to monolayer cultures, the CTG method can also be used to assess viability in spheroids [183].

The number of cells can be quantified by counting them in a microscope either by using a cell counter, or manually in a Bürker counting chamber. In order to get a more accurate count, trypan blue can be added to distinguish between living and dead/dying cells, since only the cell membranes of the latter will be permeable to the dye.

The three methods consider different aspects of a cell population, and the measured treatment effect may therefore deviate. In particular, the mechanism of action of a drug, as well as cell line variations, has been found to contribute to this discrepancy. In a study by Chan *et al.* the use of ATP and MTS-reducing assays led to an underestimation of potency and/or maximal efficacy compared to the actual number of cells present in the well, following treatment with a range of chemotherapeutic agents and kinase inhibitors [184].

6.5 Protein detection by immunohistochemistry (IHC), western blot and flow cytometry

In this thesis, different methods for studying levels and post-translational modifications of proteins have been used; immunohistochemistry (paper II), western blotting (paper I, II and III) and flow-cytometry (I and III).

Immunohistochemistry is a method that can be used for detecting a desired protein in tissue preparations and single cells. In addition to providing information on whether or not the selected protein is present, the method allows the researcher to evaluate its sub-cellular and intra-tissue localizations. Like most other assays IHC has its limitations. Tissue fixation and

processing can lead to changes in the epitopes and thereby either limit the accessibility or cause a high non-specific background, which can cause later misinterpretation of the results. Furthermore, in cohorts collect over an extended time period differences in tissue handling may occur. The subjectivity in the interpretation of staining pattern and different scoring systems can also influence the outcome [185]. The method heavily depends on the specificity of the antibody used. In paper II, we used a commercially available monoclonal Wee1 antibody (sc-5285, B-11) that was obtained from Santa-Cruz. Since the provided datasheet did not specify that the antibody could be used on IHC, precautions were therefore made to validate its specificity; first by western blot and later by evaluating staining pattern of siWee1 transfected paraffin-embedded melanoma cells. Furthermore, the IHC analysis was optimized for the antibody, and both negative and positive controls were included. The negative control included substitution of the monoclonal primary with mouse myeloma protein of the same subclass and concentration as anti-Wee1, while the positive control consisted of sections from normal placenta with known expression of Wee1.

Western blot analysis is a widely used technique for detecting a specific protein in a cell extract [186]. Proteins are first denatured and separated according to the length of the polypeptide by gel electrophoresis, transferred to a membrane and then stained with a desired antibody. Among the advantages of the method is that protein size can be detected, and shifts in the molecular size can therefore also give an indication of protein modifications. The separation of proteins by size minimizes the problem with misinterpretations due to unspecific antibody binding to other proteins. Most antibodies can be used for this method, with the exception of those that require the native conformation of the protein to be intact in order to bind. Furthermore, a single sample can be run on several gels and analyzed for multiple antibodies. Western blot as a method is, however, less suited for measuring changes in protein levels or -modifications that only occur in a small fraction of the total cell population.

Flow cytometry is a method that can be used to evaluate molecules such as proteins and DNA in cells. The technique permits the analysis of multiple parameters, gives a good quantification of signals from single cells and is thus an invaluable tool for studying sub-populations of cells. Compared to western blot analysis, the method has much lower requirements on the number of input cells, is less time-consuming and more cost-effective. Among the challenges with the method is the limited number of compatible antibodies that can be used.

In the multiparameter flow cytometry analysis performed in paper III, cells were barcoded with pacific blue, an amine reactive fluorescent dye, in order to minimize the sample to sample variation that can arise from staining variability [187]. The barcoding procedure involved labeling the different samples with increasing concentrations of pacific blue prior to combining them in one tube and staining with antibodies (pHH3 and γH2A.X) and cell cycle 633 (see below). The four populations could later be separated by their relative expression of pacific blue after the sample was run on the flow cytometer. The barcoding thus allows us to study minor changes in antibody staining in the different samples, which could otherwise be caused by differences in the relative amount of cells versus antibodies.

6.6 Measurements of cell cycle progression

Cell cycle distribution was analyzed in all three papers using flow cytometry. In paper I and II, Hoechst 33258 was used for fluorescent staining of DNA in order to determine the fraction of cells with 2n (G_1 phase), 4n (G_2 or M phase) and intermediate (S phase) DNA content. Since neither treatment with CD437 (paper I) or siRNA transfection of Wee1 (paper II) resulted in accumulation of cells with 4n DNA content, separation of G_2 and M phase cells was not performed. In paper III, a multiparameter flow cytometry analysis was conducted in order to investigate if cells were more susceptible to DNA damage, as assessed by increased expression of γ-H2A.X, in different phases of the cell cycle. In order to distinguish between cells in G_2 and M phase, those belonging to the latter phase were separated using an antibody against the phosphorylated Serine 10 residue of histone H3 (pHH3), a marker of mitosis [188]. Due to the inclusion of three additional fluorescent dyes (including pacific blue for barcoding), cell cycle 633 staining was more convenient for separating cells according to DNA content than Hoechst 33258 in this experiment. Hoechst 33258 did, however, produce DNA histograms with narrower peaks and better separation of cells according to cell cycle phase, than cell cycle 633.

6.7 Measurement of cell death and apoptosis

Cells can die in several ways, and the different forms of death can be classified according to several criteria; morphological (e.g. apoptosis, necrosis), enzymological (e.g. involvement of for instance proteases such as caspases), functional aspects (e.g. programmed or accidental) or immunological characteristics (immunogenic or non-immunogenic) [74].

Trypan blue exclusion assay was used to determine the amount of dead cells in paper I. The method can distinguish between living and dead cells based on their ability to resist uptake of trypan blue dye from the medium; however it cannot discriminate between apoptotic and necrotic cell death. Cells undergoing apoptosis *in vivo* are quickly recognized and engulfed by circulating phagocytes due to the exposure of phosphatidyl serine on the cell surface, prior to cell membrane permeabilization which could potentially cause harm to the nearby tissue [189]. When apoptotic cells take up trypan blue from the medium, it is usually due to cells undergoing a process of 'secondary necrosis' as a result of not being removed/eaten [75]. Determination of cell death by loss of plasma membrane integrity can underestimate the amount of apoptotic cells, as these could be dying without having concluded their demise [74].

Another way to study cell death is to look for nuclear fragmentation (karyorrhexis), a morphological feature of apoptosis [75]. Immunofluorescence microscopy of cells stained with Hoechst 33342, a fluorescent dye that binds to AT-rich regions of DNA, was conducted in paper I to study the occurrence of 'apoptotic bodies' following treatment with CD437.

Degradation of nuclear DNA into mono-and oligionucleosomal units is among the key biochemical features of apoptosis, and can be detected by the aid of commercial kits such as the 'Cell death detection ELISA^{plus}' kit used in paper I and II. In addition to detecting the intracellular content of histone-associated-DNA-fragments, the ELISA kit also allows detection of these in the extracellular fluid, which is referred to as a 'necrotic index' in paper I. In retrospect, a different label, such as 'presence of extracellular histone-associated DNA-fragments', would be more correct when referring to the latter. As previously mentioned, late-apoptotic cells die by a process of secondary necrosis in vitro which involves spillage of the intracellular content into the extracellular fluid, and the occurrence of histone-associated DNA-fragments in the medium could therefore result from both necrotic and formerly apoptotic cells.

Another approach to study the presence of apoptotic cells is to demonstrate activation of specific caspases. Activation of effector caspases associated with apoptosis, such as caspase 3, can be detected by Western blot analysis by cleavage products from their zymogene form.
Apoptosis mediated by caspases involves cleavage of several proteins that are required for cellular functions and survival [190]. Among the downstream targets of caspase 3 is PARP (PARP-1), a protein with multiple cellular functions including DNA-repair [81]. Cleavage of PARP by caspases results in formation of two cleavage products; a 24kDa and an 89kDa fragment [191]. Caspase-mediated cleavage of PARP is considered a hallmark of apoptosis [81], and can be detected by Western blot analysis. Cleavages of caspase-3 and PARP were used to look for apoptosis in all three papers.

6.8 Analysis of drug combinations

Drug interactions were quantified by the Chou-Talalay method in paper I (CD437 and Lexatumumab) and paper III (MK1775 and AZD7762). The method is based on the median-effect equation which is derived from over 300 rate equations of enzyme dynamics [192]. A computer program (CalcuSyn) can be used in order to calculate a combination index (C.I.) values for the drug combinations based on the Chou-Talalay method [193]. The C.I. value depicts the interaction of the combined drugs; Synergy (C.I. < 1), additive effect (C.I. = 1) and Antagonism (C.I. > 1). The Chou-Talalay method is widely used for determining drug interactions, but other systems also exists, such as Peckman's isobolograms and the response surface model [194].
The prerequisite for the Chou-Talalay method is a dose-effect curve for each drug that must contain a minimum of three data points, and give a good linear correlation of the median-effect plot (r > 0.9 in cell culture experiments). Furthermore, both drugs must have effect as single-agents. In paper III, drug interactions were determined in several cell lines using the same concentrations of drugs. In terms of abiding to method requirements, this posed a problem in two cell lines (WM983B and WM1366) where the median-effect plot for the drugs gave a less than optimal linear correlation. Drug concentrations were thus adjusted for these cell lines to allow calculations of C.I. values. Further increasing the concentrations of MK1775 and AZD7762 to a level where the inhibitors are likely unspecific, could cause a potential bias in the experiment.

7. RESULTS AND DISCUSSION

7.1 Treatment of advanced melanoma

Advanced melanoma has over the past decades proven notoriously resistant to therapy, and the survival rates of patients diagnosed with this type of cancer have subsequently been low. In the recent years, new treatment alternatives have been developed leading to improved clinical outcome for patients [52]. These approaches are mainly focused on targeting the immune system or - specific genetic mutations. Although the new drugs have led to improved overall survival, all are associated with development of resistance and severe adverse effects in patients. In the three papers included in this thesis, we have investigated the anti-tumour effect of targeting DDR mechanisms (Paper II and III) and retinoid treatment in combination with a TRAIL agonist (Paper I) in malignant melanoma. Additionally in paper II, Wee1 was examined as a potential biomarker.

7.2 Elucidation of the anti-tumour effect of the synthetic retinoid CD437 in melanoma

We initially started working with CD437 as a part of a research project that was focused on identifying new downstream targets of the MAPK/ERK signaling pathway, among which we found Nur77 (unpublished results). CD437 has been shown to up-regulate Nur77, a nuclear orphan receptor that is suggested to be involved in retinoic induced apoptosis [133,195,196]. Since treatment with CD437 had an anti-proliferative effect in melanoma cells, this spurred us to further investigate its effect in two cell lines; FEMX-1 and WM239 (Paper I).
CD437 has previously been reported to have anti-tumour effect in a range of cancer cell lines, including melanomas [131,132,197-199]. In these studies, as well as ours, retinoid treatment led to both apoptosis and cell cycle arrests. The mechanisms through which this is induced have been reported as varied and appear to be cell line-dependent, as previously described in the introduction part of this thesis. Thus, we sought to investigate some of the mechanisms underlying the anti-tumour effect of CD437, in order to potentially find rational combinations with other drugs.

In both cell lines, the protein expressions of p53 and its downstream target p21 were increased following treatment with CD437. In line with these results, the cell cycle distribution shifted in response to retinoid exposure, with an accumulation in G_1/S and S- phase of WM239 and FEMX-1 cells, respectively. Increased expression of γ-H2A.X, indicative of DNA damage, was also observed in both cell lines following exposure to CD437 (data not shown). p21 has previously been shown to induce G_1/S arrest in response to DNA damage in both a p53-dependent and –independent manner, but is according to some studies not implicated in the S-phase checkpoint [120]. As opposed to this, other studies have indicated a role of p21 and p53 in the intra-S and G_2/M checkpoints [200,201]. The relative contribution of p21 to the S-phase arrest observed in FEMX-1 cells exposed to CD437 thus remains unclear.

In a previous study, CD437 was shown to promote apoptosis through ER-stress induced genes in ovarian adenocarcinoma [202]. In these cell lines, siRNA mediated knockdown of CHOP, a central protein in ER-mediated apoptosis and -cell cycle arrest [203], impaired CD437-induced growth inhibition. In line with this study, transcriptional up-regulation of CHOP was observed in WM239 cells, and to a minor degree in FEMX-1 cells, following retinoid treatment. These results suggest that CD437 induces ER-stress in melanoma cells, a finding which can potentially be exploited for combinational therapy. For instance, a recent study showed that ER-stress induced autophagy could overcome B-RAF inhibitor resistance in melanoma [204].

Furthermore, a lysosomal pathway for CD437-induced apoptosis has been described in human leukemia cells, in which cathepsin D and free radicals act as death mediators [134]. In this study, inhibition of cathepsin D (by pepstatin A) blocked CD437-mediated generation of reactive oxygen species (ROS) and apoptosis. Increased level of cathepsin D was observed following treatment with CD437; however intracellular translocations were not assessed in our study.

CD437-induced apoptosis differed in the two melanoma cell lines in regards to caspase-dependence and DNA-fragmentation. Whereas induction of apoptosis following retinoic treatment could be abrogated by using a pan-caspase inhibitor (Z-VAD-fmk) in FEMX-1 cells, this was not the case in WM239 cells. Caspase-dependent apoptosis in response to treatment with CD437 has previously been reported in other cell systems such as human leukemia [205] and non-small cell lung carcinoma [206]. Caspase-independent apoptosis, on the other hand, has been implicated in human prostate carcinomas following exposure to the retinoid [207].

Unlike WM239 cells, DNA-fragmentation, one of the biochemical hallmarks of apoptosis [208], was not observed in treated FEMX-1 cells. One of the central endonucleases responsible for creating the characteristic DNA fragmentation pattern seen in human apoptotic cells is DFF40, a protein that under normal circumstances is kept inactive through an inhibitory binding to DFF45. Upon caspase-mediated cleavage of the latter protein, DFF40 is released and causes nuclear DNA degradation [80]. Cleavage of DFF45 was not observed in FEMX-1 cells, supporting the lack of DNA fragmentations in this cell line following treatment with CD437.

Previous studies on cells treated with CD437 have shown that apoptosis can be initiated through the extrinsic pathway leading to cleavage of caspase 8, and this was also observed in our experiments [209,210]. The mechanisms underlying these observations are, however, unclear. In a study by Boisvieux-Ulrich *et al.*, CD437 mediated caspase 8 cleavage appeared to be dependent on JNK signaling, however the mechanisms behind this phenomenon was not elucidated any further [209]. Another possible explanation is that increased expression of death receptors following retinoid treatment may sensitize cells to death signals. In line with the latter hypothesis, increased expression of death receptors has been observed in cancer cell lines treated with CD437, including ours (DR5 and Fas) [207,210,211]. Moreover, exposure to DNA-damaging agents (e.g. doxorubicin, methotrexate) have been shown to enhance expression of both Fas receptors and –ligands, resulting in activation of the extrinsic pathway of apoptosis [212].

7.3 Combinational treatment and therapeutic relevance of CD437 and Lexatumumab

The increased expression of death receptors (Fas and DR5) following treatment with CD437 prompted us to combine the retinoid with a TRAIL receptor-2 agonist, Lexatumumab. The latter agent is a human agonistic monoclonal antibody designed to activate DR5 receptors and has been shown to selectively target cancer cells, whilst generally sparing normal cells [213].

In line with what has been observed by others [149], treatment with the TRAIL agonist decreased the viability in both cell lines as a single agent. When combining Lexatumumab with CD437, the cell viability was further reduced and resulted in a synergistic effect. Supporting these results, a previous study by Sun *et al.* similarly showed an increased effect of combined treatment with TRAIL and CD437 in human lung cancer [210].

Despite causing some reduction in viability at the given concentration, cleavage of caspase 8 was surprisingly not detected in either cell line following administration of Lexatumumab as a single agent, or in combination with CD437. Likewise, cleavage of caspase 3 was not observed in FEMX-1 cells exposed to mono-or combined treatment with the drugs. Cleavage of the latter caspase was, however, seen in WM239 cells following dual-agent treatment, as opposed to mono-treatment. It is possible that the method is not sensitive enough to detect cleavage of caspases at the low concentration of drugs used in the experiments. In retrospect, it would be beneficial to substantiate the results by verifying the experiments with other methods, in particular the lack of caspase 8 activity. In both cell lines, PARP cleavage increased following the drug combination, as opposed to either single-agent treatment. This indicates an increased initiation of apoptosis, and corresponds well with the synergistic effect on viability observed after dual administration, as opposed to either CD437 or Lexatumumab as single-agents.

Retinoids regulate many cellular processes, including cell growth, differentiation and death, and have emerged as potential therapeutic agents for cancer. Several retinoids are now included in treatment of cancers, such as ATRA (acute promyelocytic leukemia)[129] and Bexarotene (cutaneous T-cell lymphoma) [214].

Although originally developed as topical treatment for acne, CD437 has shown strong apoptotic properties in a range of neoplastic cell lines of different histological origin, as well as in *in vivo* models [199,215]. Sun *et al.* previously showed that CD437 could induce apoptosis in human lung cancer, whilst sparing normal lung epithelial cells [216]. However, in another study the retinoid induced apoptosis in normal rabbit tracheal epithelial cells, indicating that adverse effects may arise from treatment [217]. Another potential drawback is drug solubility, as CD437 is highly lipophilic and thus dissolves poorly in water [218]. The latter issue could be resolved through encapsulation of the retinoid in a water-soluble host molecule, such as β-cyclodexin, as shown by Mishur et al.[218]. CD437 has not been included in clinical trials; however it serves as a prototype for a promising class of synthetically developed retinoid related molecules [219].

Clinical phase I studies have shown that Lexatumumab is well tolerated in patients, although the anti-cancer response has been limited in these studies [220-222]. It has since then been suggested that patients should be screened for tumour expression of DR5 receptors prior to treatment. Moreover, studies should be made to evaluate if TRAIL administration should be given combined- or sequentially with other drugs [223].

7.4 Wee1 protein expression increases during disease progression

In paper II, we examined Wee1 protein expression in a panel of benign nevi, primary- and metastatic melanoma, and found that the kinase expression increased in the direction of malignancy. This relationship may be explained by augmented proliferation of malignant cells, as enhanced Wee1 activity and -protein levels have been found in S- and G_2 phases of the cell cycle [93,224]. In line with this, Cyclin A and Ki67 showed a positive correlation with Wee1 in the examined specimens, indicating that these melanoma samples were more proliferative [225]. Shortly after paper II came out in 2012, a similar study to ours was published by Bhattacharya *et al.* demonstrating increased protein expression of Wee1 in primary-, as compared to metastatic melanomas (10 tumours in each group) [226]. Both studies were performed with the same Wee1 antibody. The exact reason to why the results differed remains unknown. We can, however, speculate on whether the variation in sample-size in the two studies, or perhaps the treatment status prior to the biopsy, could influence the outcome. If the metastatic melanoma samples were collected post-chemotherapy, this could potentially affect the proliferation status and hypothetically the expression of Wee1. High expression of Wee1 (protein or mRNA) has also been reported in other cancer forms, such as osteosarcoma [227], glioblastoma [228], breast cancer [229] and vulvar squamous cell carcinoma [230], while low expression has been found in non-small-cell lung cancer [231]. Furthermore, our study revealed that high expression of Wee1 was associated with thicker tumours, ulceration and decreased relapse-free survival. In agreement with these results, a large gene expression profiling study (472 tumours/502 cancer-related genes) previously showed that increased gene expression of Wee1 correlated with shorter relapse-free survival of melanoma patients [232]. In the context of the well-known role of Wee1 in DDR, its association with malignancy in melanoma and other cancer forms indicates that the kinase is necessary in order to maintain some level of genetic stability during malignant transformation.

7.5 Targeting Wee1 in melanoma

The up-regulated expression of Wee1 and its association with malignancy prompted us to study the effect of siRNA mediated knockdown of the kinase in malignant melanoma cell lines (paper II). Our hypothesis was that if Wee1 expression in malignant cells is high in order

to prevent DNA damage from occurring during cell division, removal of the kinase should potentially allow genotoxic insults and increased cell death even in the absence of exogenous insults.

Transfection with siRNA targeting Wee1 successfully reduced the kinase expression in three melanoma cell lines, WM239, WM45.1 and LOX. In accordance with our hypothesis, DNA damage, decreased viability and apoptosis were observed in WM239 and WM45.1 cells in the absence of Wee1. Similar findings have also been observed in other cancers such as sarcoma [233], colorectal and non-small-cell lung cancer [234]. The anti-tumour effect of mono-targeting Wee1 in melanoma cell lines is likely a result of a two-step process. Wee1 has previously been shown to regulate CDK2 activity during S-phase, and thereby prevent excessive initiation of replication forks which could otherwise lead to nucleotide shortage and Mus81-mediated DNA damage [157]. In line with this, DNA damage has been shown to arise in the absence of Wee1 in other studies, as well as ours [233,234]. Secondly, the kinase has an important function in the G_2/M checkpoint, and prevents mitotic entry of cells with DNA damage [92,93]. Thus, targeting Wee1 is believed to indirectly cause DNA damage and thereafter allow the cell to pass the G_2/M checkpoint. Once the cell reaches G_1, DNA DSB could be corrected by the error-prone NHEJ mechanisms, or alternatively the damaged cell may pass on to S-phase if the checkpoint is impaired. Proliferative cells can thereby accumulate DNA damage in the absence of Wee1, which potentially promotes genetic instability and/or cell death.

Surprisingly, little or no effect was observed in LOX cells deprived of Wee1. Despite removal of the kinase, $pCDK1^{tyr15}$ remained unaltered in the latter cell line, suggesting that other mechanisms may contribute to regulate this phosphorylation apart from Wee1. Myt1 is a negative regulator of CDK1/CDK2 that has a stronger preference for the tyr14 residue, but is also capable of phosphorylating the tyr15 residue of CDKs [235]. Although no further studies were performed to investigate the mechanisms underlying the persisting $pCDK1^{tyr15}$ in LOX cells in the absence of Wee1, we speculate if Myt1 could have a more prominent role in this cell line. In this regard, Guertin A *et al.* recently found that tumour cell lines with low Myt1 (PKMYT1) mRNA levels appeared to have a greater sensitivity to Wee1 inhibition [234]. Furthermore, simultaneous Myt1 knockdown in cell lines that were less responsive to Wee1 inhibition potentiated the effect of MK1775 (Wee1 inhibitor) by nearly a 5-fold. The lack of response to Wee1 removal could thus be a result of such compensatory mechanisms controlling CDK1/2 activity in LOX cells, a phenomenon which might have implications for Wee1 as a possible therapeutic target, and therefore warrants further investigation.

Several studies have found that *TP53*-mutated cells are in particular sensitive to Wee1 inhibition when combined with DNA-damaging agents or radiotherapy [179-181,236,237]. This phenomenon might be explained by *TP53* mutated cells being more reliant on the G_2/M checkpoint in order to repair DNA damage that occurs in the absence of Wee1, than cells with functional p53 that can arrest in G_1/S. As opposed to this hypothesis, mono-targeting of Wee1 has shown cytotoxic effect in sarcoma cells in a p53-independent manner [234]. In accordance with the latter study, siRNA mediated knockdown of Wee1 in WM239 ($p53^{wt}$) and WM45.1 ($p53^{Mut}$) cells resulted in approximately the same reduction in viable cells. In this regard, it should be noted that while p53 mutational inactivation is relatively rare in melanoma, its function is often abnormal [121,122]. However, whereas WM45.1 cells accumulated in S-phase in the absence of Wee1, WM239 cells were retained in G_1 whilst expressing increased levels of p53 and p21. This indicates that while the G_1/S checkpoint appears functional in WM239 cells, they still accumulate DNA damage and die in the absence of Wee1. Our results from paper II thus appear to point to a less important role of the *TP53* mutational status in terms of predicting the effect of targeting Wee1 in this cell line. However, when more melanoma cell lines were included in paper III, inhibition of Wee1 (MK1775) led to a stronger reduction in viability in *TP53* mutated cells (WM45.1 and WM983B) compared to those with wild-type (FEMX-1, WM239, patient 3 and WM1366). Moreover, in addition to $TP53^{mut}$, both WM983B and WM45.1 cells harbour mutations in the *CDKN2A* gene that encodes the CDK inhibitor proteins p14 and p16 that are involved in cell cycle regulation in G_1/S [238]. Although the relative contribution of the *TP53* and *CDKN2A* mutations in sensitizing the WM983B and WM45.1 cell lines to targeting Wee1 has not been determined, it seems plausible that the G_1/S checkpoint will be less functional in these cells.

Past studies have also indicated that the level of genetic instability is a predisposing factor to the cells sensitivity to inhibition of Wee1 or Chk1 [239,240]. In this regard, melanomas display a high degree of chromosomal instability [241], and is also one of the cancer forms harbouring most mutations [2]. In a previous study, Iorns *et al.* demonstrated that cell lines with high expression of Wee1 were in particular sensitive to inhibition-, as well as siRNA mediated knockdown of the kinase [229]. As opposed to this, no major differences in Wee1 protein level were detected in the cell lines in paper II or III.

7.6 Increased anti-tumour effect of combined targeting of Wee1 and Chk1/2

Inhibition of Wee1 has successfully been combined with for instance DNA-damaging agents [242], Heath Shock protein 90 inhibitors [243] and more recently targeting of other central proteins in DDR, such as Chk1 [244,245]. In line with this, our next approach was to combine a Wee1 inhibitor (MK1775) that is currently included in clinical trials, with a Chk1/2 inhibitor (AZD7762) in paper III. Whereas both inhibitors decreased the viability in our panel of melanoma cell lines, a stronger reduction was observed when combining them. To further assess the drug interaction we used the Chou-Talalay method for calculating combination index values, and found synergistic effects from co-targeting Wee1 and Chk1/2. In line with these results, previous studies have observed synergy when combining inhibitors of these kinases in several cancer forms, including a melanoma cell line (A2058) [244,246-248].
The increased anti-tumour effect of combined inhibition of Wee1 and Chk1/2 were also recapitulated in 3D-models. In these experiments we found that despite drug-removal, spheroids exposed to the drug combination were unable to regain growth within the time frame of the experiment (exception being WM239 cells).

The effects of mono-and combined targeting of Wee1 and Chk1/2 were further assessed in xenograft models in paper III, and resulted in slightly lower tumour volumes in mice treated with a combination of inhibitors, as opposed to mono-targeting. For this experiment we used 'Patient 3' cells, which grew well on mice, however it was not among the most responsive cell lines in our *in vitro* experiments. The cell line was recently established from a biopsy from a patient, and is therefore assumed to have undergone less selection and hold a more authentic degree of tumour heterogeneity, as compared to the other cell lines [249]. Supporting our results, a stronger effect of co-targeting Wee1 and Chk1 has been observed in colorectal cancer - and mantel cell carcinoma xenograft models, as compared to inhibition of either Wee1 or Chk1 [248,250]. Our results did, however, not reach the statistical significance seen in the latter studies.

7.7 Mechanisms underlying the anti-tumour effect of combined inhibition of Wee1 and Chk1/2

The increased anti-tumour effect of combined targeting of Wee1 and Chk1/2, as compared to mono-targeting, spurred us to investigate some of the mechanistic backgrounds to how inhibition of these kinases affects melanoma cells.

Wee1 and Chk1/2 both regulate the cell cycle through controlling CDK activity in S-phase and in the G_2/M transition. It has previously been suggested that inhibition of Wee1and Chk1/2 cause a cytotoxic effect through hyperactivation of CDKs [156,251]. Wee1 is known to directly phosphorylate the tyr15 residue of CDK1/2, whilst Chk1/2 indirectly contributes to maintain it through inhibition of the CDC25 phosphatases. In line with this, $pCDK1^{Tyr15}$ levels were reduced following inhibition of Wee1 (and to some extent Chk1/2), and even further decreased in cells exposed to a combination of these inhibitors. Likewise, a further reduction in $pCDK1^{Tyr15}$ has been observed in other studies following co-targeting of Wee1 and Chk1 [246,247]. In one of these studies, Davies *et al.* found that the cytotoxic effects of MK1775 (Wee1 inhibitor) and AR458323 (Chk1 inhibitor) were at least partially due to hyper-activation of CDKs, as the anti-proliferative effect mediated by the inhibitors could be partially rescued by CDK inhibition (Roscovitine) [247]. The lack of response to Wee1 inhibition in LOX cells, where $pCDK^{Tyr15}$ remained unaltered; further support the notion of CDK hyper-activation as a contributing factor to the anti-tumour effect of targeting at least Wee1. The studies by others, as well as our own, thus indicate that the CDK hyper-activation is one of the underlying causes of the anti-tumour effect of inhibition of Wee1 and Chk1/2. However, if increased CDK activity was the only mechanism causing the stronger anti-tumour effect of combined targeting versus single-agent exposure, then one might expect an additive, rather than synergistic effect. Although both Wee1 and Chk1 regulate replication initiation through CDK activity in S-phase, Chk1 has multiple other targets that may influence replication fork progression and -stability [157]. Furthermore, besides its contribution to CDK regulation, Chk1 also has important roles in DNA repair and the mitotic spindle checkpoint, which may contribute to the synergistic effect observed from its combined targeting with Wee1 [163,164,252].

Whereas single-agent treatment with Wee1 and Chk1/2 inhibitors induced DNA damage, this increased following combined targeting of the kinases in WM239 cells. In support of this, Guertin *et al.* previously demonstrated that inhibition of Wee1 and Chk1 led to synergistic accumulation of DNA damage in colon carcinoma cell lines [248]. Moreover, the latter study

showed that aberrant CDK activity, resulting from combined targeting of the kinases, was required for inducing DNA damage.
In line with other studies, we found that DNA damage occurred in S-phase cells following inhibition of Wee1 and Chk1/2 in all three cell lines [156,157,248]. The latter finding is in accordance with the proposed functions of the kinases in controlling genome integrity through suppression of CDK2 activity in S-phase [253]. Thus, the increased induction of DNA damage following inhibition of Wee1 and Chk1/2 may hypothetically create a positive feedback loop leading to toxic levels of damaged DNA, since DDR mechanisms are less functional in their absence.

Furthermore, combined targeting of Wee1 and Chk1/2 led to a more pronounced abrogation of the G_2/M checkpoint, as seen by an increase of cells expressing the mitotic marker pHH3 following short-time inhibition of the kinases, as compared to treatment with either single-agent. A proportion of these cells contained intermediate amount of DNA equivalent to S-phase, indicating that these had undergone premature mitosis. Similar observations have been made following inhibition of Wee1 and Chk1 in a previous study [246].

Whereas reduced viability was observed following mono- and combined inhibition, the relative effect varied in the different melanoma cell lines, with the most prominent effect observed in WM45.1 and WM983B cells. This divergent response to Wee1 and/or Chk1/2 inhibition may have several causes as previously discussed in terms of Wee1 inhibition, one of which being *TP53* and *CDKN2A* mutations. In this regard, the most responsive cell lines were also the ones with mutations in the mentioned genes, unlike the rest of the cell lines. As opposed to this hypothesis, Guertin *et al.* found that the p53 status could not predict synergy following combined inhibition of Wee1 and Chk1 in a panel of various cancer cell lines [248]. More studies should be performed in order to establish the p53-dependency, as this could potentially be an inclusion criterion for such a treatment regimen in the future.

7.8 Effect of targeting Wee1 and Chk1/2 on normal cells

The ultimate goal of cancer therapy is to selectively kill cancer cells, whilst simultaneously sparing normal dividing cells in the body. Traditional chemotherapies act on rapidly dividing cells, which unfortunately also include some of the normal cells in the body. In fact, in many organs, normal cells proliferate at the same rate or even more rapidly than tumour cells [254], such as hematopoietic cells in the bone marrow [255,256], and epithelial cells in the mucosa of the gastrointestinal tract [257], skin and hair follicles [258]. This poses a problem in terms of adverse effects when treating cancer.

To study the effect of combined inhibition of Wee1 and Chk1/2 on normal cells, we included two primary cell lines established from non-cancerous skin, FF144 SC (fibroblasts) and FOMA4 (melanocytes), in paper III. Compared to melanomas, these cells only displayed a very modest reduction in viability from combined targeting of Wee1 and Chk1/2. Similar observations have also been made in other preclinical studies, demonstrating that normal cells are less responsive to combined targeting of these kinases than cancer cells [246-248]. Moreover, mice treated with inhibitors appeared healthy in our experiments, although adverse effects were not extensively studied. The differences in response can potentially be explained by tumour cells having elevated replication stress due to genetic alterations and -instability, thereby making them more dependent on Wee1 and Chk1/2 as compared to normal cells [259,260]. Variances in proliferation rates between normal- and melanoma cells may also influence their relative sensitivity to inhibition of these kinases, making the slower dividing cells less susceptible.

7.9 Wee1 and Chk1/2 as targets for therapy

The small-molecular inhibitor of Wee1, MK-1775, has been included in several phase I studies on patients with solid- and hematologic cancers; however few study results have been published (ClinicalTrials.gov). Phase II studies are currently recruiting or ongoing. Such experimental programs have, on the other hand, been terminated for the Chk1/2 inhibitor, AZD7762, due to adverse effects in patients [261,262]. Likewise, clinical trials including other inhibitors of Chk1 and Chk1/2 have also been terminated. Despite this, phase I studies with newer agents targeting these kinases are still ongoing or recruiting. In this regard, the

reported toxicities observed in patients have appeared drug-specific, rather than class-specific, indicating that they could instead be off-target effects (reviewed in [263]). Still, concerns have been made regarding Chk1 as potential target for therapy, as the mechanisms underlying its function in normal cells are currently poorly understood [264].

Mak *et al.* recently raised another concern in terms of single-agent targeting of Wee1 or Chk1, as administration of low concentration drugs had a proliferative effect in non-small cell lung carcinoma and cervical cancer [265]. This observation is likely explained by the shortening of the cell cycle following partial deprivation of the kinases, indicating that these cells are tolerant to some degree of DNA damage or chromosomal instability associated with acceleration of the cell cycle. These limitations were, however, overcome by co-targeting Wee1 and Chk1.

8. CONCLUDING REMARKS

New treatment alternatives for patients with metastatic melanoma have emerged over the past few years leading to improved overall survival. However, all of these are associated with adverse effects, development of resistance and there are still no curative treatment alternatives for the majority of patients with advanced disease. In light of being among the cancer forms with the most increasing incidence rates, the search for additional treatment alternatives for this patient group is of great importance.

In this thesis we have investigated the anti-tumour effect of treatment with a vitamin A derivative, the synthetic retinoid CD437, in melanoma. Administration of the retinoid caused cell cycle arrest and apoptosis, and led to up-regulation of death receptors (FAS and DR5) in two cell lines. A synergistic decrease in viability was observed after combined treatment with Lexatumumab, a TRAIL agonist that activates DR5 receptors. Our results, in line with what has been reported by others, are of potential clinical interest if the drug solubility issues with CD437 can be overcome.

DNA damage response proteins, such as Wee1 and Chk1/2, have become attractive targets for therapy in the recent years. In this thesis, we have shown that the Wee1 protein expression increases in the direction of malignancy, and is associated with shorter relapse-free survival of patients. We have also demonstrated that inhibition of Wee1 and Chk1/2 result in anti-tumour effects in cell lines and xenograft model, effects which were amplified by combined targeting of the kinases. These are findings with possible therapeutic relevance, in particularly since inhibitors of Wee1 and Chk1/2 are currently included in clinical trials.

9. FUTURE PERSPECTIVES

To further evaluate the potential of Wee1 and Chk1/2 as therapeutic targets, we are currently investigating the response to mono-and combined inhibition of the kinases in primary cell lines of metastatic melanomas collected from patient biopsies, whilst also screening these samples for mutational status of *tp53* and *CDKN2A*. Determining the protein expression of Wee1 and Myt1 in these samples may also be of interest. By comparing the response to treatment one may get a better understanding of which patients could benefit from such treatment regimens. Our preliminary results are so far promising in terms of anti-tumour effect, however increased proliferation has been observed in two patient samples following targeting of Chk1/2. The latter finding emphasizes the necessity of further molecular sub-classification of the responders and defining some inclusion criteria for patients prior to treatment.
Due to the adverse effects observed in patients treated with AZD7762, we will in our future experiments substitute it with another Chk1/2 inhibitor, LY2606368, which is currently included in clinical phase II trials (ClinicalTrials.gov).

Furthermore, targeting Wee1 in combination with DNA damaging agents has led to increased anti-tumour effect in several cancer forms [179,242]. In a recent study, combined inhibition of Wee1 and the genotoxic agent doxorubicin was shown to increase DNA damage in melanoma cell lines, as compared to mono-treatment with either drug [266]. To further explore the potential in such a treatment regimen, we wish to combine Wee1 inhibition with other DNA damaging agents such as Dacarbazine.

It has been previously been demonstrated that upon inhibition of checkpoint kinases, tumour cells may activate compensatory survival mechanisms, such as the MAPK/ERK and PI3K/Akt signalling cascades, thus decreasing the therapeutic effect [267]. In line with this, we have observed that inhibition of Wee1 has led to increased activation of the MAPK/ERK signaling pathway in several melanoma cell lines. This finding spurred us to combine MK1775 with a B-RAF inhibitor (Vemurafenib), from which we found a synergistic decrease in cell viability. In our future studies, we wish to further explore the potential in this treatment combination in expanded panels of cell lines, and in xenograft models.

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11. ABBREVIATIONS

AIF	Apoptosis-inducing factor
AJCC	American Joint Committee on Cancer
Akt	V-Akt murine thymoma viral oncogene homolog
Apaf-1	Apoptosis protease activating factor 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
Bad	Bcl-2-associated death promoting homolog
Bcl-2	B-cell lymphoma-2
Bcl-xl	Bcl-2 like 1 protein (long form)
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 like 11 protein
B-RAF	v-raf murine sarcoma viral oncogenes homolog B1
CAK	CDK-activating kinase
CD437/AHPN	6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid
CDC25	Cell division cycle 25
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
Chk1/2	Checkpoint kinase 1/2
CHOP	C/ERB homologous protein
CKI	CDK inhibitor protein
CTG	Cell Titer Glo
DcR1/2	Decoy Receptor 1/2
DDR	DNA damage response
DISC	Death inducing Signaling complex
DNA	Deoxiribonucleic acid
DR4/5	Death Receptor 4/5
DSB	DNA double-strand breaks
EndoG	Endonuclease G
ERK	Extracellular Regulated Signaling Kinase
FADD	Fas-associated death domain protein

Fas	TNF receptor superfamily, member 6
FDA	Food and Drug Administration
GNA11	Guanine nucleotide binding protein (G protein), alpha 11
GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide
GRIN2A	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A)
HR	Homologous recombination
IAP	Inhibitor of apoptosis
IHC	Immunohistochemistry
IL-2	Interleukin-2
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MEK1/2	Mitogen/Extracellular signal-regulated Kinase 1/2
MITF	Microphthalmia-associated transcription factor
MOMP	Mitochondrial Outer Membrane Permeabilization
MRN	Mre11, Rad50, Nbs1
mRNA	messenger ribonucleic acid
MTS	3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl) -2H-tetrazolium
Myt1	Myelin transcription factor 1
NHEJ	Non-homologous end-joining
N-RAS	Neuroblastoma RAS viral oncogenes homolog
PARP	Poly (ADP-ribose) polymerease
PD-1	Programmed cell death 1 protein
PI3K	Phosphoinositide 3 kinase
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
Puma	p53 upregulated modulator of apoptosis
RNA	Ribonucleic acid
RPA	Replication Protein A
siRNA	Small interfering RNA

SMAC/DIABLO	Second mitochondria-derived activator of caspases/Direct IAP-binding
SSB	Single-Stranded breaks (SSB)
ssDNA	Single-stranded DNA
TNFalpha	Tumour necrosis factor-alpha
TRADD	Tumour necrosis factor receptor type 1-associated death domain
TRAIL	Tumour necrosis factor (TNF)-related apoptosis-inducing ligand
UV	Ultraviolet
XIAP	X-linked inhibitor of apoptosis protein
XP	Xeroderma pigmentosum
Yes1	Yamaguchi sarcoma viral oncogene homolog 1

I

II

High Expression of Wee1 Is Associated with Poor Disease-Free Survival in Malignant Melanoma: Potential for Targeted Therapy

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Abstract

Notoriously resistant malignant melanoma is one of the most increasing forms of cancer worldwide; there is thus a precarious need for new treatment options. The Wee1 kinase is a major regulator of the G_2/M checkpoint, and halts the cell cycle by adding a negative phosphorylation on CDK1 (Tyr15). Additionally, Wee1 has a function in safeguarding the genome integrity during DNA synthesis. To assess the role of Wee1 in development and progression of malignant melanoma we examined its expression in a panel of paraffin-embedded patient derived tissue of benign nevi and primary- and metastatic melanomas, as well as in agarose-embedded cultured melanocytes. We found that Wee1 expression increased in the direction of malignancy, and showed a strong, positive correlation with known biomarkers involved in cell cycle regulation: Cyclin A ($p<0.0001$), Ki67 ($p<0.0001$), Cyclin D3 ($p=0.001$), $p21^{Cip1/WAF1}$ ($p=0.003$), p53 ($p=0.025$). Furthermore, high Wee1 expression was associated with thicker primary tumors ($p=0.001$), ulceration ($p=0.005$) and poor disease-free survival ($p=0.008$). Transfections using siWee1 in metastatic melanoma cell lines; $WM239^{WTp53}$, $WM45.1^{MUTp53}$ and LOX^{WTp53}, further support our hypothesis of a tumor promoting role of Wee1 in melanomas. Whereas no effect was observed in LOX cells, transfection with siWee1 led to accumulation of cells in G_1/S and S phase of the cell cycle in WM239 and WM45.1 cells, respectively. Both latter cell lines displayed DNA damage and induction of apoptosis, in the absence of Wee1, indicating that the effect of silencing Wee1 may not be solely dependent of the p53 status of the cells. Together these results reveal the importance of Wee1 as a prognostic biomarker in melanomas, and indicate a potential role for targeted therapy, alone or in combination with other agents.

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Introduction

Malignant melanoma is the second most increasing form of cancer in Norway, following prostate (men) and lung cancer (women) [1]. Whereas the prognosis is good when detected early, there are no curative treatments once the cancer has spread to distant organs (stage IV). Thus, there is a desperate need for new and more effective treatment options.

The cell cycle is the orderly series of events leading to cell division, and is regulated by the assembly and activation of complexes of CDKs and cyclins, which again triggers the transition between each of the four phases; Gap 1 (G_1), DNA synthesis (S), Gap 2 (G_2) and mitosis (M). During these events, DNA damages may arise both as a consequence of normal metabolic activity and due to environmental factors, and division may be arrested/delayed at three major DNA damage checkpoints (G_1/S, intra- S and G_2/M) before cell division. The G_1/S checkpoint is largely controlled by p53, a tumor suppressor protein which function is impaired/lost in the majority of cancers, thus compromising this checkpoint. Hence most cancer cells exposed to DNA-damage rely on the S- and G_2/M checkpoints for repair to occur. Encountering the S-phase checkpoint, genomic insults cause cells to slow down cell cycle progression rather than being arrested, rendering the G_2/M checkpoint to ultimately halt the cell cycle progression [2]. Central in regulating the transition between the G_2 and M phases is Wee1-like protein kinase (Wee1), a tyrosine kinase [3]. Wee1 negatively regulates entry into mitosis by phosphorylating the Tyr15 residue of Cyclin Dependent Kinase 1 (CDK1, also known as CDC2), thus inactivating the CDK1/cyclin B complex and arresting the cell cycle.

In addition to being a key regulator of the G_2/M checkpoint, Wee1 also plays an active role in stabilizing the genome in the S-phase. By suppressing CDK2 activity during DNA synthesis, Wee1 prevents unscheduled initiation of replication that may potentially lead to DNA lesions [4].

Kinases, such as Wee1, represents potential therapeutic targets, however, their expression varies in different types of tumors. Over-expression of Wee1 has previously been reported in osteosarcoma, glioblastoma and breast cancer [5–7]. Under-expression, on the other hand, has been described in non-small-cell lung cancer [8]. Cell lines showing an enhanced level of Wee1 have also been demonstrated to be more sensitive to treatment with siWee1 [6].

Due to many promising *in vitro* results, the Wee1-inhibitor MK1775 have very recently been included in two phase I clinical trials both as mono-therapy and in combination with either 5-fluorouracil [9] or topotecan/cisplatin [10].

In the present study, we demonstrate for the first time that Wee1 is up-regulated in human malignant melanomas as compared to normal melanocytes and benign nevi, and that high expression of Wee1 is associated with poor disease-free survival and markers of increased tumor cell proliferation. Our *in vitro* results further revealed a reduced amount of viable cells, accumulation of cells in G_1/S or S-phase and double-strand DNA breaks following transfection with siWee1 in both p53 wild-type and mutated melanoma cell lines. Together our results indicate a role of Wee1 in proliferation and genomic stability in malignant melanoma, thus potentially making the kinase an eligible therapeutic target.

Materials and Methods

Speciments

Formalin-fixed, paraffin-embedded tissue sections from 108 primary malignant melanomas (75 superficial spreading (SSM) and 33 nodular melanomas (NM)), 23 metastases and 10 benign nevi (7 combined, 2 combined+intradermal and 1 intradermal) were randomly collected from the archives of The Norwegian Radium Hospital and regional hospitals. Clinical follow-up was available for all patients. The Regional Committee for Medical Research Ethics South of Norway (S-06151) and The Social and Health Directorate (06/2733) approved the current study protocol.

Immunohistochemical analysis

Three-µm sections made from formalin-fixed paraffin embedded tissues were immunostained using the Dako EnVision™Flex+ System (K8012, Dako Glostrup, Denmark). Deparaffinization, rehydration and target retrieval were performed in one operation in a Dako PT-link and EnVision™ Flex target retrieval solution with high pH. To block endogenous peroxidase the sections were treated with Dako EnVision Peroxidase Block for 5 minutes. Sections were incubated with monoclonal Wee1 antibody (B-11, sc-5285, 1:300, 0.67 µg IgG_1/mL) from Santa Cruz Biotechnology, Inc.(CA, USA) for 30 minutes. Thereafter, the sections were incubated with Dako EnVision™ FLEX+ mouse linker for 15 minutes followed by incubation with Dako EnVision™ FLEX/HRP for an additional 30 minutes. For visualization of staining, the sections were treated with 3′3-diaminobenzidine tetrahydrochloride (DAB) Chromogen (Dako), counterstained with haematoxylin, dehydrated and mounted from xylol with Richard-Allan Scientific Cyto seal XYL (Thermo scientific, MA, USA). Sections from normal placenta with known expression of Wee1 was used as positive control, whereas negative controls included substitution of monoclonal antibody with mouse myeloma protein of the same subclass and concentration as anti-Wee1. Four semiquantitative classes were used to describe the number of stained tumor cells: absent, 0; $<10\%$, 1; 10–50%, 2; $>50\%$, 3. Staining in cytoplasm and nucleus were evaluated separately. Wee1 expression in more than 10% of the tumor cells was considered as high. The expression pattern of Wee1 was compared to proteins previously examined in our melanoma panel, where high expression has been set as $>5\%$: Cyclin A [11], Ki67 [11], Cyclin D3 [12], Cyclin D1 [12], p27 [13] $p21^{CIP1/WAF1}$ [14] and p53 [15].

Statistical analysis

Statistical analysis was performed using of SPSS version 18.0 (Chicago, IL). The relationship between the expression level of Wee1 and tumor thickness was evaluated non-parametrically using the Mann-Whitney two sample test. Comparison between Wee1 expression and Ki-67, Cyclin A,- D1,- D3, $p21^{CIP1/WAF1}$, $p27^{kip1}$, p53 as well as SSM and NM, was conducted by the use of chi-square tests. Kaplan-Meier survival estimate was used to evaluate the impact on survival.

Melanocytes isolation

Normal melanocytes were isolated from human foreskins. Briefly, foreskins derived from circumcisions of newborns were washed with Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). Excess adipose tissues were removed, and the skin specimens were cut into approximately 0.5×0.5 cm^2 pieces and incubated in 0.48% dispase II (Invitrogen) at 4°C. After 18 hours, the epidermis was manually removed from the dermis, cut and digested in 0.05% trypsin for 5 min in 37°C. The suspensions were diluted in 254CF medium (Invitrogen) and serially filtered through 40 µm cell strainers (Becton Dickinson, Franklin Lakes, NJ). The cells were plated in the T25 flask and cultured until confluence was reached. Differential trypsinization was used for first passage in order to obtain a pure melanocyte culture. Once confluent, 2×10^6 melanocytes were harvested using EDTA, embedded in 200 µL 1.5% agarose and fixed in 10% neutral buffered formalin for 1 hour, and processed by routine histological methods.

Cell lines and Growth conditions

The human metastatic melanoma cell lines WM45.1 and WM239 were kindly provided by Dr. Meenhard Herlyn (the Wistar institute, Philadelphia, USA) [16,17]. The LOX cell line was established from a lymph node biopsy of a melanoma metastasis, at the Norwegian Radium Hospital (Oslo University Hospital, Norway) [18]. All cell lines were maintained in RPMI-1640 medium (LONZA, Verviers, Belgium) supplemented with 5% Fetal Calf Serum (Biochrom, KG, Berlin, Germany) and 2 mM L-glutamine (LONZA, Verviers, Belgium). The cells were grown in monolayer culture at 37°C in humidified conditions containing 5% CO_2 and 95% air.

Small interfering RNA (siRNA) transfection

All cell lines were plated out in either 6-well plates (1.5×10^5 cells/well) or in 96-well plates (5×10^3 cells/well) 24 hrs in advance of the transfection. The cells were transfected with 10 nM siRNA targeting Wee1 (OligioID; 'VHS50841') or RNAi negative control duplexes (Negative Control LOW GC, 12935-200) using Lipofectamine™ RNAiMAX transfection reagents (all reagents from Invitrogen corporation, CA, USA).

MTS assay

Five thousand cells per well were seeded in 96-well plates and left to attach overnight, before siRNA transfection for the indicated time.

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (Promega, WI, USA), in which the capacity of the cells to convert MTS salt into a brown formazan product was measured. Absorbance was measured at 490 nm using ASYS UVM340 96-well plate reader.

Trypan blue dye exclusion test

Cells treated with SiCtr or SiWee1 were harvested using trypsin/EDTA (LONZA), along with medium containing floating cells. After centrifugation, the cell pellet was resuspended in PBS containing trypan blue (Merck, Stockholm, Sweden). Viable (dye excluding) and trypan blue stained dead cells were counted.

Cell Death Detection ELISA^{plus}

Determination of cytoplasmic histone-associated-DNA-fragments was assessed using a commercially available kit (Roche Diagnostic GmbH, Mannheim, Germany), following the manufacturers instructions. The presence of histones in cytoplasm is indicative of apoptosis. The ELISA signal was quantified by measuring the absorbance at 405 nm (reference 495 nm), using ASYS UVM340 96-well plate reader (Fisher Scientific, Oslo, Norway).

Flow cytometric cell cycle analysis

Cells were harvested by trypzination and washed 1× in PBS. Cell pellets containing approximately 10^6 cells were re-suspended in 1 mL 70% ice-cold methanol and left to fixate for a minimum of 24 hrs. Fixated cells were washed 1× in PBS, and stained with a solution containing 2 µg/mL Hoechst 33258 in PBS. Flow cytometric analysis was performed using LSR II UV laser (BD biosciences, San Jose, CA).

Western blot analysis

Cells were harvested using a rubber policeman, washed once in 1×PBS, and then lysed in ice-cold NP-40 Lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF), Aprotenin (0.02 mg/mL), Phosphatase inhibitor cocktail 1 (10 µL/mL), Phosphatase inhibitor cocktail 2 (10 µL/mL), PhenylMethaneSulfonyl Fluoride (PMSF) (1 mM), Leupeptin (0.02 mg/mL), Pepstatin (0.02 mg/mL) and Sodium vanadate (1 mM) (Sigma-Aldrich, St. Louis, MO)). Bradford (Bio-Rad Laboratories AB, Sundbyberg, Sweden) analysis was performed for protein quantification, and 25 µg protein/lane was resolved in SDS polyacrylamide gel electrophoresis (PAGE) and transferred to a PDVF immobilon membrane (Millipore, Bedford, MA). To ensure even loading, filters were stained with naphtholblue black (Sigma-Aldrich) and later re-stained with α-tubulin. The membranes were blocked in 5% non-fat milk in TBST (150 mM NaCl, 25 mM Tris-Cl, (pH 7.5), 0.01% Tween 20), and probed with primary antibodies at 4°C overnight, with gentle agitation. Primary antibodies Caspase 3 (#9662/#9664 (even mix)), Caspase 8 (#9746), Caspase 9 (#9502), Cyclin B1 (#4138S), Cyclin D3 (#2936), $p21^{CIP1/WAF1}$ (#2946), p-p38 Thr180/Tyr182 (#4631) and PARP (#9532), were purchased from Cell Signaling (Beverly, MA). α-tubulin (DMIB) was acquired from Calbiochem (Nottingham, UK), whereas Cyclin A (sc-751), p53 (sc-126) and Wee1 (sc-5285) were obtained from Santa Cruz (Santa Cruz, CA). γ-H2AX (#05-636) and $pCDK1^{Tyr15}$ (ab47594) antibodies were acquired from Millipore and Abcam (Cambridge, England), respectively. Membranes were thereafter washed 3×10 minutes in TBST. Membranes were hybridized with an appropriate secondary antibody (HPR-conjugated anti-rabbit or anti-mouse IgG antibodies (Promega)) for 1 hr at room temperature, with gentle agitation, and then washed in TBST for 3×10 minutes. Protein bands were detected after first incubating the membranes with ECL-plus (GE Healthcare, Chalfont St Gils, UK) for 5 minutes, and then exposing them to X-ray films.

Results

Increased expression of Wee1 in melanoma

High protein expression of Wee1 has previously been reported in human cancers [5–7]. Since the status of Wee1 expression in melanomas has not been extensively studied, paraffin-embedded tissue from a panel of benign nevi and primary- and metastatic melanomas, in addition to a sample of cultured melanoncytes, were analyzed for Wee1 protein expression by immunohistochemisty. As illustrated in Figure 1A, protein expression of Wee1 was hardly detectable in the nucleus of the cultured melanocytes, however brown granules were seen in the cytoplasm, most likely due to melanin. Furthermore, as demonstrated in Table 1 and illustrated in Figure 1A, a heterogeneous Wee1 staining pattern was observed in the vast majority of the tumor samples. However, the percentage of positive cells varied in tissues of different stages. Based on distribution, positive immunoreactivity in ≥10% of the tumor cells was used as cut-off to discriminate between high and low Wee1 expression. Whereas only 20% of the nevi displayed Wee1 expression in ≥10% of the tumor cells, this was the case for 42% of the primary- and 70% of the metastatic tumors. Furthermore, while none of the examined nevi contained >50% Wee1 immunoreactive cells, such expression was found in 4% of the primary melanomas and 22% of the metastatic tissues. Nodular lesions expressed higher levels of Wee1 than the superficial spreading tumors. Wee1 expression was in all cases, except two, exclusively localized to the cell nucleus.

High expression of Wee1 is associated with poor prognosis and increased proliferation

Since expression of Wee1 increased in direction from nevi to primary- and metastatic melanomas, we next examined the relationship between Wee1 expression, clinical parameters and disease outcome. As shown in Table 2, and Figure 1B, high Wee1 expression (in ≥10% of the tumor cells) was significantly associated with thicker primary tumors ($p = 0.001$), T-staging ($p = 0.004$), as well as with ulceration ($p = 0.005$) and poor disease-free survival ($p = 0.008$). No association with over-all survival was found (data not shown).

Since our panel of melanomas has been previously analyzed for other regulators of the cell cycle, we examined the relationship between Wee1 and expression of these parameters (Ki-67, Cyclin A,- D1,- D3, $p21^{CIP1/WAF1}$, $p27^{kip1}$ and p53) [11–15]. As shown in Table 2, significant co-variations between Wee1 expression and cyclin A ($p<0.0001$), Ki67 ($p<0.0001$), Cyclin D3 ($p = 0.001$), p53 ($p = 0.025$) and $p21^{CIP1/WAF1}$ ($p = 0.003$) were detected. No associations between Wee1 expression and Cyclin D1 and $p27^{kip1}$ were observed (data not shown). Together these results suggest that high Wee1 protein expression is associated with increased proliferation in human melanomas.

In vitro results support a role of Wee1 in proliferation and genome stabilization

To further study the role of Wee1 in melanomas we knocked-down its expression using siRNA in the three metastatic cell lines, WM239 (p53-wild-type), WM45.1(p53-mutated) and LOX (p53-wild-type). Wee1 was effectively silenced in all three cell lines, as confirmed by western blotting; however, phosphorylation on Tyr15 of CDK1, a downstream target of Wee1, was only down-regulated in WM239 and WM45.1 cells (Figure 2A). Decreased cell viability as estimated by MTS (Figure 2B) and a relative reduction of living cells (Figure 2C), were observed after 24, 48 and 72 hours of siWee1 transfection in WM239 and WM45.1, but not in LOX cells.

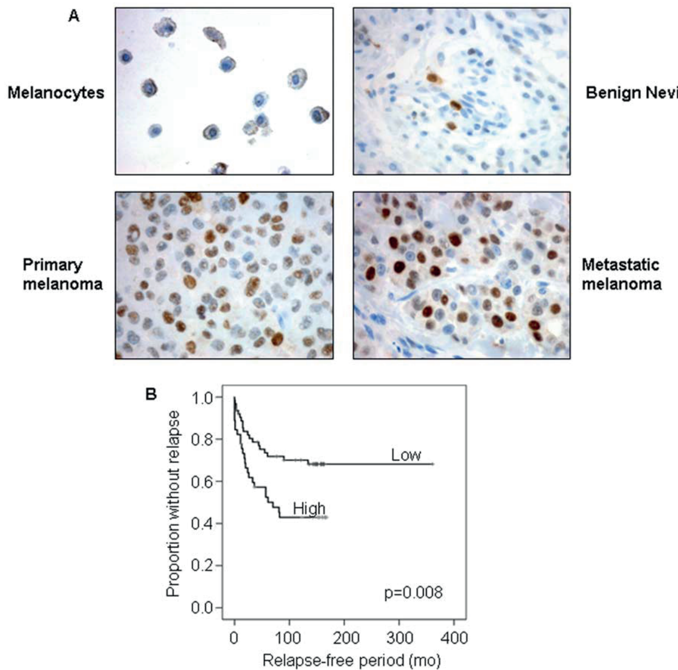

Figure 1. High Wee1 expression increases with tumor progression and is associated with a shorter relapse-free period. A. Wee1 expression in cultured melanocytes, benign nevi, primary- and metastatic melanoma, analyzed by immunohistochemistry. B. Melanoma patients were grouped according to Wee1 expression in their tumors (high (n = 44) or low (n = 63)). Relapse-free survival in months was estimated for both groups and presented as a Kaplan Meyer curve.
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Furthermore, we observed that Wee1 silencing led to increased cell death in WM 239 and WM 45.1 as determined by the cell death detection ELISA^{plus} kit (Figure 3A). Likewise, cleavage of Poly(ADP-ribose) polymerase (PARP) and pro-caspase-3, markers of apoptosis [19], were detected in the absence of Wee1 (Figure 3B).

Table 1. Number (percentage) of melanocytic lesions expressing different levels of Wee1.

Expression level		Low		High	
	No. Analyzed	**0%**	**<10%**	**10–50%**	**>50%**
Nevi	10	0 (0%)	8 (80%)	2 (20%)	0 (0%)
Primary melanoma	108	3 (3%)	60 (56%)	41 (38%)	4 (4%)
Superficial spreading	75	1 (0%)	49 (65%)	24 (32%)	1 (0%)
Nodular	33	2 (6%)	11 (33%)	17 (52%)	3 (9%)
Metastatic melanoma	23	1 (4%)	6 (26%)	11 (48%)	5 (22%)

Wee1 expressions in benign nevi, primary- and metastatic melanoma were estimated by immunohistochemistry, and categorized in four semi-quantitative classes according to percentage of immunoreactive tumor cells. The groups were further divided into low (<10%) and high (≥10%) expression.
doi:10.1371/journal.pone.0038254.t001

Serine 139 phosphorylation of H2AX (γ-H2AX) is a sensitive marker for DNA double-strand breaks, and may be constitutively expressed in untreated cells due to oxidative DNA damage during metabolic activity [20,21]. Expression of γ-H2AX was observed in all cell lines, however in the absence of Wee1, an increase of γ-H2AX was observed in WM239 and WM45.1 cells, indicating increased DNA damage (Figure 3B).

The p53 tumor suppressor protein accumulate in the presence of DNA damages, thus leading to DNA repair, cell cycle arrest or apoptosis [22]. An increase in p53 protein expression was observed in WM239 ($p53^{WT}$) cells following treatment with siWee1, but not in WM45.1 ($p53^{MUT}$) or LOX cells ($p53^{WT}$). Notably, these results indicate that inhibition of Wee1 may sensitize melanoma cell lines to DNA damage regardless of their p53 status.

Since Wee1 is a key regulator of the G_2/M phase transition, we studied the effect of Wee1 knockdown on cell cycle progression. As demonstrated in Figure 4A, flow cytometry analysis revealed accumulation of WM239 and WM45.1 cells in the G_1/S- and S-phase, respectively. The cell cycle distribution was, however, not affected in siWee1 treated LOX cells. Furthermore, immunoblotting revealed that cyclin D1, -A, and –B1 protein levels were weakly to moderately down-regulated in WM239 and WM45.1, but not in LOX cells. Moreover, a marginal decrease in cyclin D3 expression was observed in WM239 cells. Despite increased p53 expression, $p21^{CIP1/WAF1}$ protein expression was weakly increased

Table 2. Wee1 expression correlates with clinical parameters- and markers of tumor progression.

Clinical parameter	No. Analyzed	Expression	Low	High	p-value †
Mean tumor depth	105		1.99 mm	3.90 mm	0.001
T-stage					
T1 (0–1 mm)	27		22 (81%)	5 (19%)	0.004
T2 (1.01–2.0 mm)	34		21 (62%)	13 (38%)	
T3 (2.01–4 mm)	18		7 (39%)	11 (61%)	
T4 (>4 mm)	26		10 (38%)	16 (62%)	
Ulceration	100	No	46 (46%)	22 (22%)	0.005
		Yes	12 (12%)	20 (20%)	
Marker*					
Cyclin A	99	Low	39 (39%)	12 (12%)	<0.0001
		High	19 (19%)	29 (29%)	
Ki67	99	Low	46 (46%)	17 (17%)	<0.0001
		High	12 (12%)	24 (24%)	
Cyclin D3	99	Low	48 (48%)	21 (21%)	0.001
		High	10 (10%)	20 (20%)	
p21$^{Cip1/WAF1}$	71	Low	30 (42%)	9 (13%)	0.003
		High	13 (18%)	19 (27%)	
p53	67	Low	38 (57%)	20 (30%)	0.025
		High	2 (3%)	7 (16%)	

*Low expression of Cyclin A [11], Ki67 [11], Cyclin D3 [12], p21 [13] and p53 [14]; defined as immunoreactivity in <5% of the tumor cells. Wee1 expression in <10% of tumor cells is defined as low.
†Statistical significances determined by Chi-square tests.
doi:10.1371/journal.pone.0038254.t002

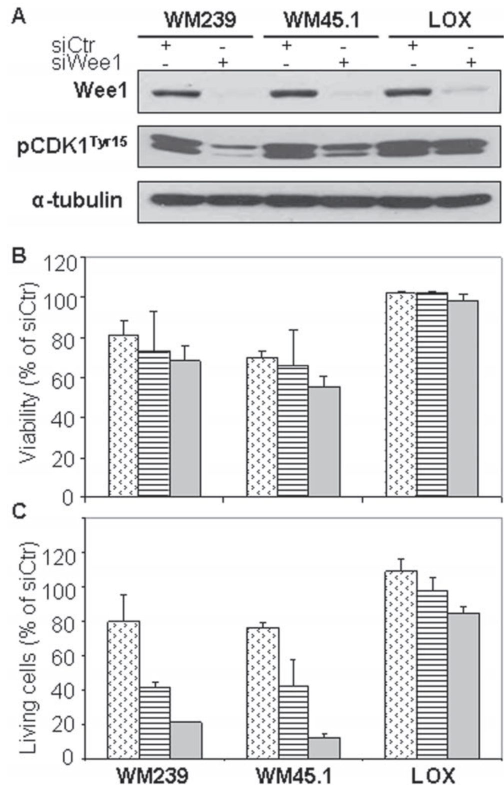

Figure 2. Transfection with siWee1 effectively shuts down Wee1 expression and reduces cell viability. A. Cells were transfected with siWee1 for 48 hours. Expressions of Wee1 and pCDK1^{Tyr15} were examined by western blot analysis. α-tubulin was used as loading control. The figure is representative of at least three independent biological experiments.B and C. Cells were transfected with siWee1 (dots: 24 h, stripes: 48 h and no-pattern: 72 h). The relative amount of viable cells was estimated by MTS (B), and the relative quantity of living cells was estimated by counting trypan-excluding cells (C).
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in WM239 cells. No alterations were seen in WM45.1 or LOX cells (Figure 4B). As previously reported, p21$^{CIP1/WAF1}$ was not constitutively expressed in WM45.1 cells, and Wee1 silencing did not affect its expression [23]. The p38 MAP kinase signaling pathway has previously been shown to be involved in p53-independent cell cycle arrest as a response to DNA damage [24], hence we next examined its activation in the absence of Wee1. In support of this hypothesis, increased phosphorylation of p38, indicative of an active signaling pathway, was observed in WM239 and WM45.1, but not in LOX cells, following transfection with siWee1.

Discussion

In the present study, immunohistochemistry was applied to examine the level of Wee1 in a panel of benign nevi and primary – and metastatic melanomas, as well as in one sample of isolated normal melanocytes, in order to evaluate the impact of altered expression on disease progression and clinical outcome. We demonstrate that Wee1 up-regulation follows tumor progression and is associated with thicker tumors, ulceration and decreased relapse-free survival. Similar results have previously been reported in other forms of human cancers, such as glioblastoma and breast cancer [5,6]. In non-small-cell lung cancer, on the other hand, reduced Wee1 expression was associated with a higher recurrence rate [8]. Furthermore, Wee1 showed a strong, positive correlation with markers of proliferation: Cyclin A, Ki67 and Cyclin D3 [25]. In support, we have previously reported that increased expression of Ki67, Cyclin A and -D3 is associated with tumor thickness, progression and poor clinical outcome in melanomas [11,12]. In line with these findings, our *in vitro* results demonstrated that in the absence of Wee1, both Cyclin D1, -D3 (only in WM239) and -A protein expression were weakly decreased in two out of three melanoma cell lines. Based on these findings, we hypothesize that Wee1 contributes to increased proliferation in melanomas.

The augmented expression of Wee1 may seem as a controversy in malignant tumors, based on its well-known inhibitory role in cell cycle progression. However, Wee1 also has a role in genomic stabilization during replication by preventing DNA damage to occur [26,27]. Furthermore, if other mutations have led to increased CDK- activity, elevated levels of Wee1 may be beneficial to avoid premature mitotic entry resulting in cell death [4]. Our *in vitro* results using siRNA mediated downregulation of Wee1, led to increased cell death, thus further emphasizing the association with malignancy observed *in vivo*. It is therefore likely that the high

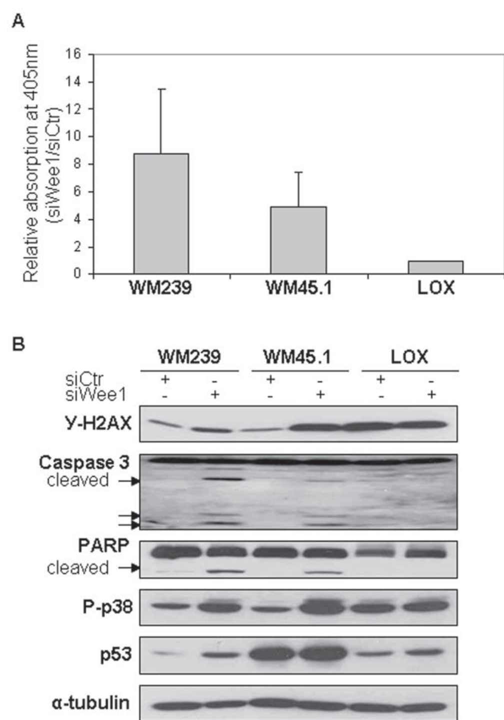

Figure 3. Transfection with siWee1 promotes DNA damages and apoptosis. A. Presence of cytoplasmic oligonucleosomes was measured by ELISA following 48 h siWee1 transfection. Induction of apoptosis shown as enrichment factor calculated as absorbance at 405 nm of siWee1 treated cells relative to siCtr treated cells. B. Protein expressions were measured by Western blot following 48 h transfection with either siCtr or siWee1. Cleavage of PARP and Caspase 3 are shown with arrows. α-tubulin was used as loading control. The figure is representative of at least three independent biological experiments.
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levels of Wee1 observed protect the melanoma cells against DNA damage and cell death. In line with this hypothesis, our *in vitro* results showed that double-strand DNA damage, as demonstrated by increased γH2AX expression, occurred in the absence of Wee1 in both WM239 and WM45.1 cells, and was accompanied by accumulation of cells in the G_1/S- and S phases, in the two cell lines, respectively. In accordance with our results, Dominiguez-Kelly *et al.* [27] recently reported augmented amounts of γH2AX in cells stalled in the S-phase, following treatment with siWee1. Given its role in maintaining genomic stability in S-phase, we speculate whether cells lacking Wee1 may fail to regulate CDK activity during replication, thus leading to DNA damage and S-phase arrest [26]. Another possible explanation may be that cells in lack of a functional G_2/M checkpoint rush into mitosis without securing a proper DNA synthesis, potentially leading to so-called mitotic catastrophe and cell death [7,28].

In accordance with a study by Hashimoto et al. [29], using Wee1 inhibitor PD0166285 in murine melanoma, silencing of Wee1 also led to decreased proliferation in WM239 and WM45.1 cells in the present study. Strikingly, the growth inhibitory effect in murine melanomas was even stronger than what was observed in our study following siWee1 transfection. However, whereas siWee1 is believed to be highly specific, PD0166585 is a nonselective Wee1 inhibitor which even at low concentrations can target a range of other kinases involved in regulating CDK activity, such as Membrane-associated tyrosine/threonine protein kinase 1 (MYT1) and Serine/threonine-protein kinase 1 (CHK1) [28,30]. The increased effect may also simply be due to the differences in tumor cell lines. Notably, silencing of Wee1 had no effect on LOX cells in terms of proliferation, cell death or cell cycle distribution. However, phosphorylation of its downstream target $CDK1^{Tyr15}$ was not abolished in this cell line, thereby providing a possible rationale for lack of response to treatment with siWee1. Hence, we speculate if other mechanisms are more central in CDK1 regulation in this cell line, for instance MYT1, known from other cell systems to have much of the same functions as Wee1 [31].

In the present study we found that Wee1 had a strong positive correlation with p53 expression and $p21^{CIP1/WAF1}$ in primary melanomas. High p53 expression has previously been shown to correlate with poor clinical outcome and increased proliferation in metastatic melanoma [32,33], however the opposite has also been found [34]. Likewise, we have previously reported that p53 protein expression is increased in metastatic melanoma compared to benign nevi, however, although not significant, high expression was also associated with a more favorable disease progression [15]. Despite being mutated in the majority of human cancers, mutational inactivation of p53 is rare in melanomas; yet the protein may not function as normal. In this regard, it was shown that despite being expressed as wild-type in melanoma, p53 could activate some genes in response to stress, but lacked the ability to inhibit growth or induce apoptosis [33,35]. Interestingly, our *in vitro* results demonstrated that the effects of silencing Wee1 were not exclusive to p53 mutated cell lines. In contrast to our findings, effects of inhibiting Wee1 in other cancer forms have in previous studies been described as limited to cells with mutated p53, in particular when combined with DNA damaging agents [36–38].

Additionally, $p21^{CIP1/WAF1}$, a down-stream target of p53, was significantly correlated with Wee1 in primary melanomas. $p21^{CIP1/WAF1}$ is a well-known inhibitor of CDKs, and is known to promote cell-cycle arrest in response to many stimuli, however the protein may also exhibit oncogenic activities [39]. In line with this, we have previously demonstrated that $p21^{CIP1/WAF1}$ expression is up-regulated in primary melanomas compared to benign nevi, and is associated with thicker tumors [14]. When silencing Wee1 *in vitro*, $p21^{CIP1/WAF1}$ expression increased marginally, in WM239 cells only, suggesting that the association between Wee1 and $p21^{CIP1/WAF1}$ observed *in vivo* could be due to indirect mechanisms. However, the accumulation of cells in G_1/S phase, accompanied by increased $p21^{CIP1/WAF1}$ protein expression, in siWee1 treated $WM239^{p53wt}$ cells, suggests that the augmented p53 protein level probably is able to trigger the G_1/S checkpoint in response to DNA damage in this cell line. Hence we speculate if the increased cell death seen in WM239 cells in the absence of Wee1, is related to the cells inability to control CDK activity during DNA replication, rather than the ability to stop the cell cycle progression in G_2/M. Notably, Reinhardt *et al.* has previously reported that activation of the p38/MAPK signaling pathway may cause cell cycle arrest after DNA damage in the absence of p53 [24]. In both WM239 and WM45.1 cells activation of the p38/MAPK signaling pathway increased in the absence of Wee1 suggesting that the p38/MAPK signaling pathway may contribute to the arrest following DNA damages induced by siWee1.

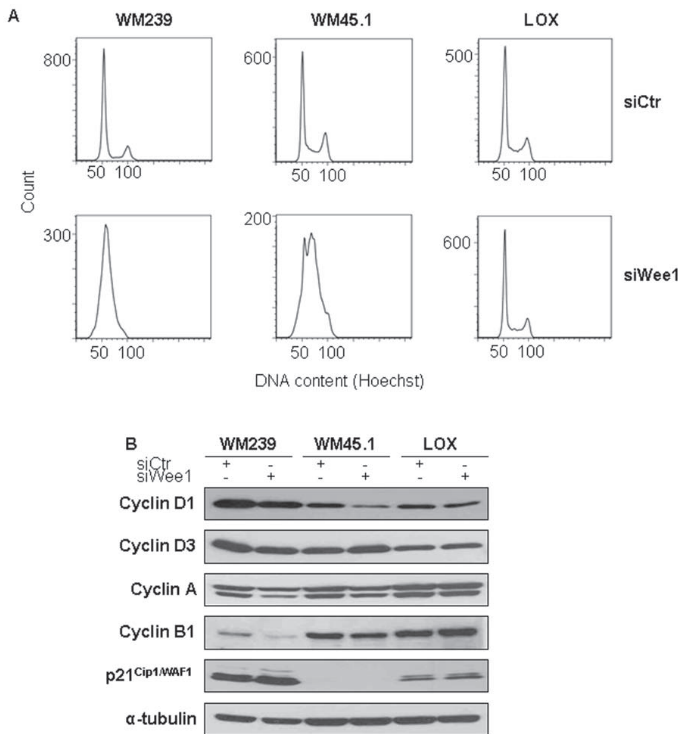

Figure 4. Transfection with siWee1 leads to alterations in cell cycle distribution (A) and -associated proteins (B). A. Histograms showing cell cycle distribution after siWee1 transfection for 48 hours, measured by flow cytometry. B. Cells were treated with siWee1 for 48 hours and protein expressions were analyzed by immunoblotting using the indicated antibodies. α-tubulin was used as loading control. The figure is representative of at least three independent biological experiments.
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In conclusion, our results indicate that despite being an inhibitor of cell cycle progression, high expression of Wee1 is associated with malignancy and poor prognosis in patients with melanoma. Our *in vitro* results further support these findings; silencing of Wee1 resulted in DNA damage and increased cell death in two out of three cell lines regardless of p53 status. Thus, high expression of Wee1 appears to protect the cancer cell from DNA damage and ultimately cell death. These findings potentially make Wee1 an eligible target in melanoma, both as mono-therapy and in combination with DNA damaging agents.

Author Contributions

Conceived and designed the experiments: VAF GIM. Performed the experiments: GIM AKRR EE AS. Analyzed the data: GIM RH. Contributed reagents/materials/analysis tools: VAF RH AS. Wrote the paper: GIM VAF.

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III

Combined inhibition of the cell cycle related proteins Wee1 and Chk1/2 induces synergistic anti-cancer effect in melanoma

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Abstract

Background: Malignant melanoma has an increasing incidence rate and the metastatic disease is notoriously resistant to standard chemotherapy. Loss of cell cycle checkpoints is frequently found in many cancer types and makes the cells reliant on compensatory mechanisms to control progression. This feature may be exploited in therapy, and kinases involved in checkpoint regulation, such as Wee1 and Chk1/2, have thus become attractive therapeutic targets.

Methods: In the present study we combined a Wee1 inhibitor (MK1775) with Chk1/2 inhibitor (AZD7762) in malignant melanoma cell lines grown in vitro (2D and 3D cultures) and in xenografts models.

Results: Our in vitro studies showed that combined inhibition of Wee1 and Chk1/2 synergistically decreased viability and increased apoptosis (cleavage of caspase 3 and PARP), which may be explained by accumulation of DNA-damage (increased expression of γ-H2A.X) - and premature mitosis of S-phase cells. Compared to either inhibitor used as single agents, combined treatment reduced spheroid growth and led to greater tumour growth inhibition in melanoma xenografts.

Conclusions: These data provide a rationale for further evaluation of the combination of Wee1 and Chk1/2 inhibitors in malignant melanoma.

Keywords

Malignant melanoma, Wee1, MK1775, Chk1/2, AZD7762, cancer therapy, cell cycle inhibitors,

Background

Malignant melanoma is the deadliest form of skin cancer, in addition to having one of the most increasing incidence rates of all cancer forms [1]. Although curable by surgical excision at an early stage, patients diagnosed with metastatic melanoma (stage IV) have had a median survival of 6-10 months [2]. Despite recent year's advances leading to new treatment options such as Ipilimumab, Vemurafenib, Trametinib and Dabrafenib, there are still no curative treatment alternatives for the majority of the patients with advanced disease [3,4].

In response to DNA-damage, the dividing cell is arrested through activation of checkpoint mechanisms in order to allow time for DNA-repair to be completed. If the damage is too severe, apoptosis or senescence is induced to ensure that unrepaired DNA-damage is not passed on to future generations of cells. Loss of checkpoint mechanisms is frequently found in cancer, a trait which can be exploited in cancer therapy. Wee1 is a kinase involved in checkpoint regulation that in response to DNA-damage or replication stress can halt the cell cycle progression in S- and G2 phases by adding inhibitory phosphorylations (Tyr15) on cyclin-dependent kinases CDK2 and CDK1, respectively [5]. In our previous study we found that Wee1 was up-regulated in human melanomas as compared to benign nevi, and that high expression of Wee1 was associated with poor disease-free survival [6]. Likewise, over-expression of Wee1 (protein and/or mRNA) has been reported in osteosarcoma, glioblastoma and ovarian- and vulvar squamous cell carcinomas, thus emphasizing its potential as a therapeutic target in cancer [6-10] Furthermore, targeting Wee1, either by siRNA mediated silencing or inhibitors, has in several studies, including ours, been shown to lead to increased DNA-damage and apoptosis [6,11,12].

Although mono-targeting of Wee1 has shown anti-tumour effect in some cancer cell lines, a stronger effect has been observed when combining Wee1 inhibitors with for instance DNA-damaging agents, Heath Shock Protein 90 inhibitors and more recently inhibitors of other cell

cycle regulatory proteins such as Chk1/2 [13-16]. The Chk1/2 kinases are key regulators of DNA-damage surveillance pathways and DNA repair. Chk2 is a protein that is stably expressed throughout the cell cycle and is activated in response to DNA-damage; in particular DNA double-strand breaks through the ATM-Chk2-p53-p21 pathway. The expression of Chk1, on the other hand, is primarily found in S- and G2 phases, where it is active even in the unperturbed cell cycle. During normal cell cycle progression, Chk1 has been shown to regulate replication forks during DNA replication and entry into mitosis. Chk1 activation in response to DNA-damage is preferentially triggered through the ATR-Chk1-CDC25 pathway, although some cross-talk between Chk1 and ATM has been reported (reviewed in [17]Chk1 has been shown to activate Wee1 in Xenopus extracts and yeast; however such a relationship has not been recapitulated in higher eukaryotes [18,19]. Previous studies have shown an advantageous effect of combining Wee1 and Chk1/2 inhibitors as compared to mono-targeting in a variety of cancer cell lines, and points to complementary functions of the kinases [13,20,21]

In the present study we investigated the combined use of the Wee1 inhibitor MK1775 with a Chk1/2 inhibitor (AZD7762) in a panel of metastatic melanoma cell lines. While both inhibitors had an effect as mono-agents, combined administration gave a stronger anti-tumour effect both in vitro and in xenografts models. Co-treatment led to increased dephoshorylation of CDK1, DNA-damage, premature mitosis and apoptosis. In summary, our results warrant further evaluation of combined use of Wee1 and Chk1/2 inhibition in malignant melanoma.

Materials and methods

Cell lines and growth conditions. The human metastatic melanoma cell lines WM239, WM45.1, WM983B and WM1366 were kindly provided by Prof. Meenhard Herlyn (the Wistar institute, Philadelphia, USA) [22,23]. The FEMX-1 cell line was established at the Radium hospital [24]. The 'Patient 3' cell line was a kind gift from Prof. Peter Hersey (Royal North Shore Hospital, Sydney, Australia) [25]. All cell lines were maintained in RPMI-1640 medium (LONZA, Verviers, Belgium) supplemented with 5% Fetal Calf Serum (Biochrom, KG, Berlin, Germany) and 2 mM L-glutamine (LONZA, Verviers, Belgium). The cells were grown in culture at 37ºC in humidified conditions containing 5% CO_2, either as monolayer cultures in 75cm2 bottles or in 96 flat-bottom well plates. Normal human melanocytes (FOMA4) and fibroblasts (FF144sc) were isolated from human foreskin and cultured in 254CF (Invitrogen corporation, CA, USA) and DMEM 10% FBS medium, respectively, as previously described [6].

Spheroids were generated by plating suspended cells (500-4000 cells/well, dependenton the cell line) in Corning® 96 Well Clear Round Bottom Ultra Low Attachment Microplates (Corning, MA, USA). Spheroid formation was allowed for 3 days prior to treatment. Images of spheroids were obtained using phase-contrast on an Olympus IX81 microscope with a 4x objective. Spheroid volume was calculated using Olympus Soft Imaging Solution Gm6H software. A minimum of two independent biological experiments were conducted, where each experiment contained at least four parallels of the individual treatment options.

Chemical inhibitors. Wee1 inhibitor MK1775 and Chk1/2 inhibitor AZD7762 were purchased from Selleck Chemicals (TX, USA) and used for time intervals and concentration indicated in the text.

Small interfering RNA (siRNA) transfection. All cell lines were plated in either 6-well plates (1.5 x 10^5 cells/well) or in 96-well plates (5 x 10^3 cells/well) 24 hrs in advance of the transfection. The cells were transfected with 10nM siRNA targeting Wee1 (OligioID; 'VHS50841'), Chk1 (OligioID: 'VHS40226') or RNAi negative control duplexes (Negative Control LOW GC, 12935-200) using Lipofectamine™ RNAiMAX transfection reagents (all reagents from Invitrogen corporation, CA, USA). Transfection was allowed for 5 hrs before the medium was replaced with RPMI w/5% FCS and 2mM L-glutamine. Transfected cells were harvested after 48 hrs for further analysis.

Viability assays. Four thousand cells per well were seeded in 96-well plates and left to attach overnight, before treatment with MK1775 and/or AZD7762 for 48 hrs. The growth inhibitory effects of mono- and combined treatments were measured using the3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (Promega, WI, USA). Absorbance was measured at 490nm using ASYS UVM340 96-well plate reader. Alternatively, viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega) following the manufacturer's protocol. Luminescence was measured using GloMax® Luminometer (Promega). Viability of treated cells was normalized to the untreated control cells. Each experiment was performed with three parallel observations and repeated at least three times.

Calcusyn analysis. Synergy was determined by the Chou and Talalay Combination Index (C.I.)[26] for non-exclusive treatments (treatments affecting different targets or sites of the same target), and calculated by Calcusyn software (BioSoft, Feruson, MO, USA). Of note, this method requires that a dose effect curve for each drug is made, in which the data-points give a good r-value (>0.90 for cell systems) [27]. Given the variation in dose effect of the drugs in the different cell lines, the concentrations of the inhibitors were adjusted for the individual cell lines (suppl. Figure 2) in order to abide to the requirements of the method

Western blot analysis. Cells were harvested and western blot conducted as previously described [6]. Caspase 3 (#9662/#9664 (even mix)), Caspase 8 (#9746), Caspase 9 (#9502), PARP (#9532) and Wee1 (#4936S) primary antibodies were purchased from Cell Signaling (Beverly, MA). α-tubulin (DMIB) was acquired from Calbiochem (Nottingham, UK), whereas Cyclin A (sc-751) and p53 (sc-126) antibodies were obtained from Santa Cruz (Santa Cruz, CA). γ-H2AX (#05-636) and $pCDK1^{Tyr15}$ (ab47594) antibodies were acquired from Millipore and Abcam (Cambridge, England), respectively. Following primary hybridization, membranes were washed 3 x 10 minutes in TBST and hybridized with an appropriate secondary antibody (HPR-conjugated anti-rabbit or anti-mouse IgG antibodies (Promega)) for 1 hr at room temperature, with gentle agitation. Membranes were then washed in TBST for 3 x 10 minutes before being incubated in ECL-plus (GE Healthcare, Chalfont St Gils, UK) for 5 minutes. Protein bands were visualized by exposing the membranes to X-ray films.

Flow cytometry and barcoding. Cells were harvested by trypzination and fixated in 70% ice-cold ethanol at -20˚C for a minimum of 24 hrs. In order to eliminate variation in antibody staining, control as well as treated cells were labelled with different concentrations of Pacific Blue (0.125, 0.031, 0.0062 and 0.00078ng/µL, respectively) for 30 min at RT in the

dark, in accordance with the fluorescent cell barcoding (FCB) technique [28]. All samples were subsequently mixed in one tube and incubated with primary antibodies (mouse anti-γH2AX (1:500, 05-636, Millipore) and rabbit anti-phospho Histone H3 (ser10)(1:500, CS#9701, Cell Signaling)) diluted in detergent buffer (0.1% Igepal CA-630, 6.5mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl, 137mM NaCl, 0.5mM EDTA [pH 7.5]) containing 4% nonfat milk for 1 hr at RT, and afterwards with secondary antibodies; anti-rabbit Alexa488 and anti-mouse Dyelight549 (1:1000, Invitrogen) for 30 min at RT in the dark. Cells were finally incubated with PBS containing Cell Cycle 633/Fx cycle™ far red stain (1µL/mL,) and PureLink RNAse A (5µL/mL) (Invitrogen) for 30min at 4˚C in the dark. Flow cytometric analysis was performed using a LSRII flow cytometer (BD Biosciences) with Diva software, and the four samples were gated based on the Pacific Blue signal before analyzes of cell cycle distribution, γH2AX and phospho Histone H3 (ser10) expression.

In vivo studies. 'Patient 3' cells ($2x10^6$) were subcutaneously injected on each side of the dorsa of nude female mice (athymic nude foxn1 nu) and tumour bearing mice were subsequently divided into groups of 7 mice each. MK1775 and AZD7762 were dissolved and administered as recommended by the manufacturer (Selleck Chemicals). Briefly, 30 mg/kg MK1775 were given orally whereas 25mg/kg AZD7762 were administered intravenously both as single agents and in combination. The treatments were given three times a week for two weeks. Tumour sizes were measured regularly using a calliper, and the volume V was calculated as follows: $V = W^2 \times L \times 0.5$ (where W and L are tumour width and length, respectively). The experimental protocol was evaluated and approved by the National Animal Research Authority and conducted in accordance with regulations of the European Laboratory Animals Science Association.

Statistical Analysis. All statistical analyzes were conducted using SPPS PASWStatistics version 18. Comparison of tumour volume at day 48 was performed with one-way between-groups ANOVA. Post-hoc comparisons were performed using the Tukey HSD tests.

Results

Synergistic effect of combined treatment with Wee1 inhibitor MK1775 and Chk1/2 inhibitor AZD7762 in melanomas

In order to study the effects of targeting Wee1 and Chk1/2, a panel of six metastatic melanoma cell lines were treated with increasing concentrations (0,063µM to 1µM) of the commercially available inhibitors MK1775 (Wee1) and AZD7762 (Chk1/2) for 48 hrs (Figure 1A and 1B). Mono-treatment with either inhibitor reduced the viability in all tested cell lines, though the effect differed in a dose- and cell line dependent manner. The most pronounced effect was observed in WM45.1 (IC50: 0.4µM (MK1775), 0.15µM (AZD7762)) and WM983B (IC50: 0.35µM (MK1775), 0.04µM (AZD7762)) cells. In the other cell lines neither MK1775 nor AZD7762 were able to reduce the viability by 50% following treatment with concentrations up to 1µM of either drug (Figure 1A and 1B).

We next investigated if combining the drugs could further decrease the cell viability as compared to single-agent treatments. The concentrations of the inhibitors used for combinational studies were chosen to be within the target-specific range of the compounds [29,30]. While exposure to either 200nM MK1775 or 100nM AZD7762 for 48 hrs only led to a modest reduction, co-treatment resulted in a marked reduction in cell viability (Figure 1C). For WM983B cells, however, 100nM AZD7762 alone decreased the viability by 90% and co-treatment with MK775 did not enhance the effect. When reducing the AZD7762

concentration, combined treatment with MK1775 was superior to single-agent treatment also in this cell line (Suppl. Figure 2). Of note, primary cultures of melanocytes (FOMA4) and fibroblasts (FF144SC) were only modestly affected when exposed to either mono- or combinational treatment (Figure 1C). Finally, in order to verify that the effect of the inhibitors could be assigned to targeting Wee1 or Chk1 we performed siRNA mediated knockdown of the proteins in three of the cell lines (WM239, WM45.1, WM983B) (Suppl. Figure 1A and 1B).

To further address the anti-tumour effect of combining the two inhibitors, drug interaction was determined using the Chou-Talalay method for quantifying synergy or antagonism [26]. Co-treatment with different concentrations of MK1775 and AZD7762 resulted in Combination Index (C.I.) values of less than 1 in the majority of cases, indicating a synergistic interaction of the drugs (Table 1 and Suppl. Figure 2).

Together, our results demonstrate an increased anti-tumour effect of co-targeting Wee1 and Chk1/2 in melanoma cell lines.

Combined inhibition of Wee1 and Chk1/2 leads to reduced viability and irreversibly inhibits growth of melanoma 3D-cultures

Three-dimensional tumour cell cultures have in several studies been reported as superior to monolayer cultures in terms of reflecting in vivo conditions for testing drug delivery systems and efficacy [31-33]. Hence, our next aim was to study long-term effects of MK1775 and AZD7762 on cells grown as spheroids (3D-cultures). Cells cultured as spheroids for three days were exposed to 200nM MK1775 and/or 100nM AZD7762 for a total of 3 days, and followed for an additional 8 days (Figure 2A). During the latter period, 50% of the medium was replaced twice with drug-free medium. Whereas spheroids given mono-treatments appeared diminished on day 14, combinational inhibition led to a marked decrease in spheroid

size (Figure 2B and 2D). Moreover, daily monitoring of growth revealed that WM45.1 and WM983B spheroids treated with the drug combination were unable to expand in size following drug-removal, thus indicating an irreversible effect within the time-frame of the experiment (Figure 2D). The volume of the WM239 spheroids, however, continued to increase following treatment, but remained markedly reduced compared to the control. In consistence with this, cell viability estimated by CellTiterGlo assay on day 14 was greatly reduced in spheroids treated with the drug-combination in contrast to those in the control or mono-treatment groups (Figure 2C). In accordance with our observation in 2D models, the decrease in viability following combinational treatment was more profound in WM45.1 and WM983B spheroids, as compared to WM239 cells. Together, our results indicate that combined use of MK1775 and AZD7762 has an increased anti-tumour effect compared to mono-treatment also in 3D cultures, and prevents spheroid growth even after drug-removal.

Combinatorial therapy reduces tumour growth in melanoma xenografts

Based on the promising in vitro results, we next investigated the in vivo efficacy of treatment with MK1775 and AZD7762 in melanoma xenografts. For this study we chose a cell line named 'Patient 3', that was recently established from a metastasis from a patient undergoing treatment with the selective BRAF inhibitor PLX4720, and previously DTIC [34]. Mono-treatment with either inhibitor resulted in reduction of tumour sizes as compared to the control group, and a tendency to a more prominent effect in the group given a combination of the two drugs was observed (Figure 3). A statistically significant difference ($p<0.05$) in tumour volume was found by ANOVA. Post-hoc comparisons using the Tukey HSD test indicated that the mean score of the control group ($M=14.47$, $SD=2.87$) was significantly different ($p < 0.024$) from the combination group ($M=6.47$, $SD=3.88$). No direct immediate toxic effects were observed during the experiment with the given dosages.

Inhibition of Wee1 and Chk1/2 lead to DNA-damage and apoptosis

To elucidate the mechanisms by which MK1775 and AZD7762 affect melanoma cell viability, we first examined the effect on Tyr15 phosphorylation of CDK1 in WM239, WM45.1 and WM983B cells after being exposed to 200nM MK1775 and/or 100nM AZD7762 for 48 hrs. Whereas Wee1 inhibition decreased pCDK1 expression in all cell lines, the combination of drugs resulted in an even stronger reduction, as assessed by western blot analysis (Figure 4A). Down-regulation in pCDK1 expression following treatment was also observed after shorter exposure times (1, 3, 7 and 24 hrs, data not shown). Interestingly, treatment with the Chk1/2 inhibitor, alone or in combination with Wee1 inhibition, decreased the total level of CDK1 in WM983B cells. Similar patterns were observed following siRNA transfections targeting Wee1 and Chk1, although combinational treatments with siRNA had less effect than what was observed with the inhibitors (Suppl. Figure 1C).

Furthermore, as seen in all three cell lines, both mono- and combinational treatment, as well as siRNA mediated knockdown, led to increased expression of γ-H2A.X, indicative of DNA double-strand breaks [35] (Figure 4A and Suppl. Figure 3C). A further increase in γ-H2A.X was observed in WM239 cells following combined targeting of Wee1 and Chk1/2, as opposed to treatment with either inhibitor alone. Cleavages of Caspase 3 and PARP, associated with apoptosis, were observed in all three cell lines following treatment with the inhibitors. Combined inhibition led to a more pronounced cleavage product when compared to either single agent treatment, as assessed by immunoblotting (Figure 4A). These findings indicate that simultaneous targeting of Wee1 and Chk1/2 leads to DNA-damage and apoptosis in melanoma cell lines.

Wee1 and Chk1/2 inhibitors induce premature mitosis and DNA damage in S-phase cells

To study how MK1775 and AZD7762 affect cells in different phases of the cell cycle, treatment with the respective drugs were allowed for three hours (Figure 4B-E and Suppl. Figure 3). The short time frame serves as a compromise between allowing the drugs to work, while at the same time limiting cell cycle progression. Cells in different phases of the cell cycle were gated based on their relative amount of DNA and expression of the mitotic marker phospho-Histone H3 (pHH3), as analyzed by flow cytometry (Figure 4B). Narrow gating for cells belonging to G1, S and G2 were used in order to avoid incorrect/uncertain assumptions of which cell cycle phase a cell belongs to (Suppl. Figure 3). Higher drug concentrations (2μM MK1775/1μM AZD7762) were used given the short exposure time, (Figure 4B-E), however the same trends were also observed following treatment with lower doses of MK1775 (200nM) and AZD7762 (100nM) (Suppl. Figure 4). Whereas exposure to either inhibitor led to an increased amount of mitotic cells, this fraction was augmented by the drug combination. Moreover, combined treatment led to an increased fraction of S-phase cells co-expressing pHH3, indicative of cells having entered mitosis prematurely (Figure 4B, 4C and Suppl. Figure 4).

To further study how the inhibitors affect cells in different phases of the cell cycle, we examined the median expression of γ-H2A.X in the gated sub-groups (Figure 4C, (for gating of cells, see Suppl. Figure 3)) following short-time drug exposures. As seen in figure 4D and 4E, increased expression of γ-H2A.X was predominantly found in S-phase cells, indicating that cells undergoing DNA-replication are especially sensitive to DNA-damage. Our results therefore suggest that combined treatment with MK1775 and AZD7762 leads to premature mitosis and DNA-damage in S-phase cells.

Discussion

In our previous study we showed that high expression of Wee1 is associated with poor disease-free survival in patients with melanoma and that in vitro targeting of the kinase leads to decreased cell viability. To further evaluate Wee1 as a potential target in melanoma, we have in the present study combined a Wee1 inhibitor (MK1775) with a Chk1/2 inhibitor (AZD7762). Our hypothesis is that simultaneous inhibition of two cell cycle control proteins will introduce a high degree of DNA-damage incompatible with cell viability. The combined inhibition led to an increased anti-tumour effect both in vitro and in xenografts models. Furthermore, our in vitro studies showed that co-treatment synergistically decreased viability and increased apoptosis, which may be explained by DNA-damage- and premature mitosis of S-phase cells. Our results provide a rationale for further testing of the treatment regimen in melanoma models.

Mono-targeting of Wee1, either by siRNA mediated knockdown or inhibitors, has shown a potent anti-tumour effect in some cancer cell lines [6,36] but only limited effect in other studies [7,8]. In line with this, the efficacy of mono-targeting of Wee1 by MK1775 varied in the tested melanoma cell lines in the present study, where low concentrations (200nM) had little effect in some of the cell lines, such as WM1366. Interestingly, a stronger anti-tumour efficacy was observed in the same cell line following exposure to MK1775 in a recent study by Haarberg *et al.*[37], however the experimental conditions in our studies differ with regard to exposure time to the drug and assay for detecting viability.

For the purpose of increasing the efficacy, inhibition of Wee1 (MK1775) has been combined with other therapeutic agents in clinical trials, such as gemcitabine and cisplatin [ClinicalTrials.gov]. Simultaneous inhibition of Wee1 and Chk1, on the other hand, has only been investigated in preclinical studies, but has been found as an eligible combination following siRNA high-throughput screens of acute myeloid leukemia, lung-, prostate- and

ovarian cancer [13,20,21]. Combined inhibition of Wee1 and Chk1 using commercial inhibitors have been shown to synergistically enhance the therapeutic efficacy in several cancer cell lines, including the A2058 melanoma cell line [13,16,20,38]. In agreement with these reports, combined targeting of Wee1 and Chk1/2 led to a synergistic reduction of viability in our cohort of melanoma cell lines. In addition, the same trends were observed for combined siRNA mediated knockdown of Wee1 and Chk1 compared to mono-targeting of either protein. However, siRNA mediated down-regulation and use of small molecular inhibitors are not always comparable [21]. The experiments using siRNA only targeted Chk1, whereas AZD7762 inhibits both Chk1 and Chk2. Still, a previous study by McNeeley *et al.* showed that the anti-tumour activity of AZD7762 following DNA damage is likely related to Chk1- rather than Chk2 inhibition [39].

Similar to what was observed in monolayer cultures, an increased effect of combined targeting was also found in multicellular spheroids. Likewise, a weak increase in efficacy was observed in melanoma xenografts following combined treatment versus either mono-treatment alone. A previous study showed that combinational inhibition of Wee1 (MK1775) and Chk1 (MK8776) led to increased in vivo efficacy in neuroblastoma xenografts [40], providing additional support for our results.
Studies have indicated that the response to Chk1 or Wee1 inhibitors is dependent on the p53 mutational status of the cells, in particular when combined with DNA-damaging agents, while others have reported no such effect [41,42]. Partly supporting the first hypothesis, the anti-tumour effect was most pronounced in the p53 mutated WM983B and WM45.1 cells, and less in the p53WT cell line WM239. Although disabling point mutations in *TP53* are only found in 10% of melanomas, inactivation of the protein is found in approximately 90% of the tumours (reviewed in [43]), suggesting that the effect of checkpoint inhibition in melanoma is

not strictly dependent on the p53 mutation status. Furthermore, the level of genetic instability has been suggested as a predictor of the response to Wee1 and Chk1/2 inhibition [44,45]. In this regard, melanomas have been shown to be one of the most genetically unstable tumour forms [46,47]. Combined inhibition of Wee1 and Chk1/2 in primary cultures of melanocytes or fibroblasts had less effect on viability than observed in the melanomas. In accordance with these results, Carrassa *et al.* found no synergistic effects of combining Wee1 (MK1775) and Chk1 inhibitors PF-00477736 (or allegedly AZD7762) in the normal fetal human lung cell line MRC-5 [20]. While both Wee1 and Chk1 are known to have functions in the unperturbed cell cycle, normal cells are less proliferative and have a lower degree of genetic alterations- and instability than tumour cells, which may explain the difference in response to kinase inhibition. However, phase I clinical trials with AZD7762 have been terminated due to cardiac toxicity in patients [48,49], and also clinical trials with other Chk1 and Chk1/2 inhibitors (reviewed in [50]) have been terminated. Concerns have been raised on whether targeting Chk1 may lead to toxicity in normal cells due the multiplicity of functions of the protein in the unperturbed cell cycle (Reviewed in [51]). Furthermore, embryonic lethality is associated with Chk1 depletion in mice [52]. On the other hand, the observed toxicities in patients following treatment with various Chk1 inhibitors have appeared as drug-specific rather than class-specific and may thus be a consequence of off-target effects (reviewed in [50]). Clinical studies with novel Chk1 (MK-8876/SCH 900776) and Chk1/2 (LY2606368) inhibitors with improved specificity are currently ongoing or recruiting (ClinicalTrials.gov).

When further investigating the effect of combined inhibition of Wee1 and Chk1/2, we found a stronger reduction in pCDK1 (tyr15) levels compared to treatment with either drug alone. In line with this, Wee1 has been shown to directly phosphorylate CDKs, and removal of Wee1 is thus expected to reduce such phosphorylations [53]. Chk1, on the other hand, phosphorylates the CDC25 phosphatases, leading to the latter proteins sequestration or

degradation, thereby hindering activating dephosphorylation of pCDK1/2 (tyr15) [54]. CDK1/2 phosphorylation is thus indirectly maintained by Chk1, and removal of the latter may promote further reduction of pCDK1/2. Inhibition of Wee1 and Chk1 has previously been shown by Davis *et al.* to be accompanied by decreased expression of pCDK1 and a synergistic reduction in cell viability [13]. They further showed that the antiproliferative effect of the inhibitors was partially reversed after CDK inhibition (Roscovitine), suggesting that CDK hyperactivity may be a contributing factor, rather than the sole cause of the antiproliferative effect of Chk1 and Wee1 inhibition. Deregulated activity of CDKs, as a consequence of Wee1 depletion, has in a previous study been shown to induce replication stress and loss of genomic integrity through subsequent nucleotide-shortage and increased firing of replication origins [55] In line with this, we observed that inhibition of Wee1 led to increased levels of DNA-damage and apoptosis, as assessed by expression of γ-H2A.X and cleavage of caspase 3 and PARP, respectively. The effect on apoptosis was amplified by combining the inhibitors, supporting a synergistic effect on viability. A further increase in DNA-damage following combined targeting of the kinases was seen in WM239 cells. Similarly, combined inhibition of Wee1 and Chk1 has in a previous study been demonstrated to induce a more intense and durable DNA-damage and anti-tumour effect than either drug alone [38]. Interestingly, treatment with the Chk1/2 inhibitor or Chk1 siRNA reduced the total protein level of CDK1 in the WM983B cell line. In agreement with this, transcriptional reduction of cell-cycle regulators, such as CDK1, has been observed after Chk1 depletion in somatic cells [56].

Similar to what has been reported by others [38,57,58], inhibition of Wee1 as well as Chk1 was shown to induce DNA-damage in the S phase of the cell cycle. These findings are in accordance with the proposed function of the kinases in restraining CDK activity during DNA replication (reviewed in [59]). Furthermore, it has been suggested that the cytotoxic effect of

Chk1 inhibition in melanoma cells is most likely due to inhibition of Chk1 in S phase, which drives cells prematurely from late S phase into an aberrant mitosis [44]. In line with this, combinational treatment with Chk1/2 and Wee1 inhibitors increased the population of S-phase cells co-expressing the mitotic marker, pHH3. These are cells that have entered mitosis without having completed replication, most likely due to the compromised G2/M checkpoint in the absence of Wee1 and/or Chk1. Likewise, an increased proportion of mitotic cells was observed following mono-targeting, and to a further extent by the combination of the inhibitors. Most likely the G2 cells have abrogated the G2/M checkpoint and entered mitosis given the short timeframe of this experiment (3 hrs).

In summary, our results support an increased anti-tumour effect of combined inhibition of Wee1 and Chk1/2 and provide a rationale for further evaluation of the kinases as therapeutic targets in human melanomas.

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Figure Legends

Figure 1. Increased anti-tumour effect of combined inhibition of Wee1 and Chk1/2.

A and B, Melanoma cell lines grown as monolayer cultures were treated with increasing concentrations (0.063- 1μM) of MK1775 (**A**) or AZD7762 (**B**) for 48 hours, and viability was measured by MTS analysis. **C.** Metastatic melanoma cell lines and primary cultures of human melanocytes (FOMA4) and fibroblasts (FF144 SC) were exposed to 200nM MK1775 and/or 100nM AZD7762 for 48 hours. Cell viability was determined by MTS analysis. Data presented are the mean of at least three independent biological experiments and presented with positive standard deviation.

Figure 2. Cytotoxic effects of MK1775 and AZD7762 are not reversed by medium renewal in 3D cultures. A. Experiment outline; spheroid formation was allowed prior to treatment (day 0-3), inhibitors (MK1775: 200nM, AZD7762: 100nM) were added on day 3. On day 6 and 10

50% of the medium was substituted by fresh medium containing no inhibitors. **B.** Spheroids were visually assessed on day 14 using light microscopy; scales in bottom right corners show 500μm. **C.** Spheroid viability was estimated by CellTiterGlow analysis on day 14, and presented as the average of four individual experiments. **D.** Spheroid volume (Y-axis) was measured/estimated using Olympus Soft Imaging Solution Gm6H software. Changes in volume were estimated for each spheroid relative to its size on day 3. The graphs represent the average of two individual experiments presented with positive standard deviations.

Figure 3. Combinatorial treatment with MK1775 and AZD7762 reduces tumour growth in melanoma xenografts. Patient 3" xenografts were treated with MK1775 (30mg/kg, three times per week, orally), AZD7762 (25mg/kg, three times per week, i.v. injection) or a combination of theinhibitors, for two weeks (7 mice per group). The tumour volumes were measured twice a week using a caliper, and are presented as tumour volume relative to the volume of the tumour at the initiation of the treatment. Standard bars represent positive standard deviation. Comparison of tumour volume at day 48 was performed with one-way between groups ANOVA, and post-hoc comparisons with the Tukey HSD tests (* $p < 0.024$ untreated vs. combination group).

Figure 4. Combined inhibition of Wee1 and Chk1/2 leads to increased mitotic entry and DNA-damage in S-phase cells. A. Cells were treated with MK1775 (200nM) and/or AZD7762 (100nM) for 48 hours. Expression of proteins involved in DNA-damage response and apoptosis was determined by western blot analysis, and α-tubulin was used as loading control. **B, C, D and E.** WM983B cells were treated with either DMSO (control) or high concentrations of MK1775 (2μM) and/or AZD7762 (1μM) for 3 hours, and analyzed by Flow Cytometry **B**. Subpopulation gating of mitotic (blue) and pre-mitotic (purple) cells were based on their DNA content and expression of pHH3. The percentages of cells are indicated above the gated populations. **C**. The percentage of cells in mitosis (M) and pre-mitosis (Pre-M) are shown as fold change from the control cells. **D**. Expression of γ-H2A.X, indicative of DNA-damage, was assessed following treatment with inhibitors. **E.** The median expression of γ-H2A.X was determined for cells in the indicated phases of the cell cycle (for gating see Suppl. Figure 3), and shown as fold change from the control cells. Data are representative or average of four independent experiments.

Supplementary figures legends.

Supplementary Figure 1. Increased anti-tumour effect of SiRNA mediated knock-down of Wee1 and Chk1. A and B. Cell viability was measured 48h following SiRNA mediated knockdown by MTS (**A**) and CellTiterGlow (**B**) assays. **C**. Expression of proteins involved in DNA-damage response and apoptosis was determined by western blot analysis, and α-tubulin used as loading control. Data are the mean or representative of three independent experiments.

Supplementary Figure 2. Synergistic effect of combining Wee1 and Chk1/2 inhibitors.
A .Cells were treated with three different concentrations of MK1775 (200, 400 and 800nM) and/or AZD7762 (50, 100 and 200nM) as indicated. Cell viability was measured by MTS after 48 hours. **B.** The combination Index (C.I) values were calculated by CalcuSyn software based on the Chou Talalay method for measuring drug interaction, for each combination of inhibitors **C and D.** Drug concentrations were adjusted for WM983B (MK1775; 50, 100 and 200nM and/or AZD7762 (20, 40 and 80nM) and WM1366 (MK1775; 1, 2 and 4μM and/or AZD7762 (0.25, 0.5 and 1μM) cells in order to abide to Chou Talalay method requirements.
. Data are the mean of three independent experiments and presented with error bars showing positive standard deviation.

Supplementary Figure 3. Example of sub-gating of cells in different phases of the cell cycle. Different samples (Control, MK1775, AZD7762, Combination) were separated by their relative expression of Pacific blue (See Material and methods for barcoding procedure). Cells of different phases of the cell cycle were sub-gated based on DNA content and expression of the mitotic marker pHH3 (dark green: G1, Blue: S, orange: G2 and purple: Mitotic cells). Median expressions of γ-H2A.X were determined by Diva software.

Supplementary Figure 4. Combined inhibition of Wee1 and Chk1/2 leads to increased mitotic entry and DNA-damage in S-phase cells. Cells were treated with indicated concentrations of MK1775 (MK) and or AZD7762 (AZD) for 3 hours, barcoded and analyzed by flowcytometry. **A.** Percentage of cells in mitosis (M) and pre-mitosis (Pre-M) were calculated relative to the control. **B.** Median expression of γ-H2A.X in cells of different cell-cycle phases, relative to the control. Data are the mean of four independent experiments

Table 1. The Combination Index (C.I.) values after combined treatment with MK1775 and AZD7762

Cell line	**Average C.I. value***	**Standard deviation**
WM239	0.3	± 0.1
WM45.1	0.9	± 0.3
WM983B	0.7	± 0.4
WM1366	0.6	± 0.4
FEMX-1	0.4	± 0.1
"Patient 3"	1.0	± 0.2

*C.I. values are estimated from the dose response effects presented in Suppl. Figure 2A and 2B.

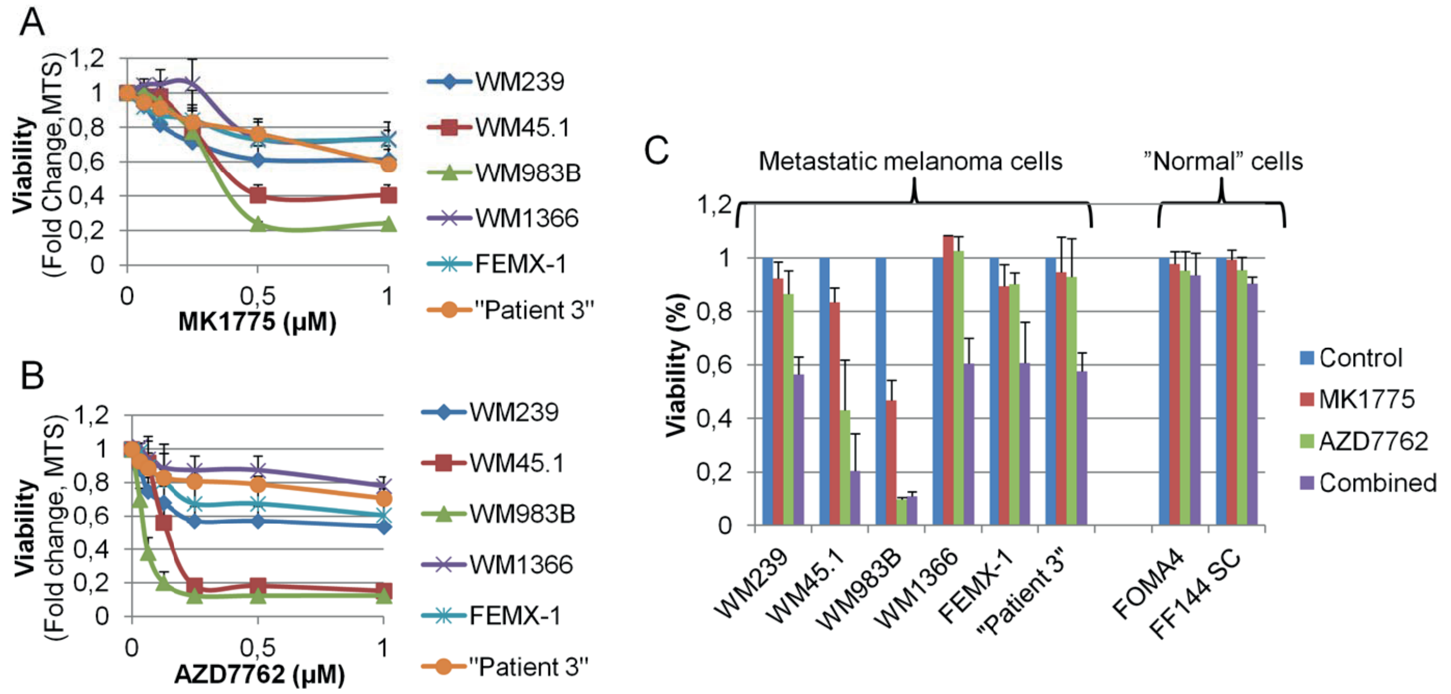


FIGURE 1

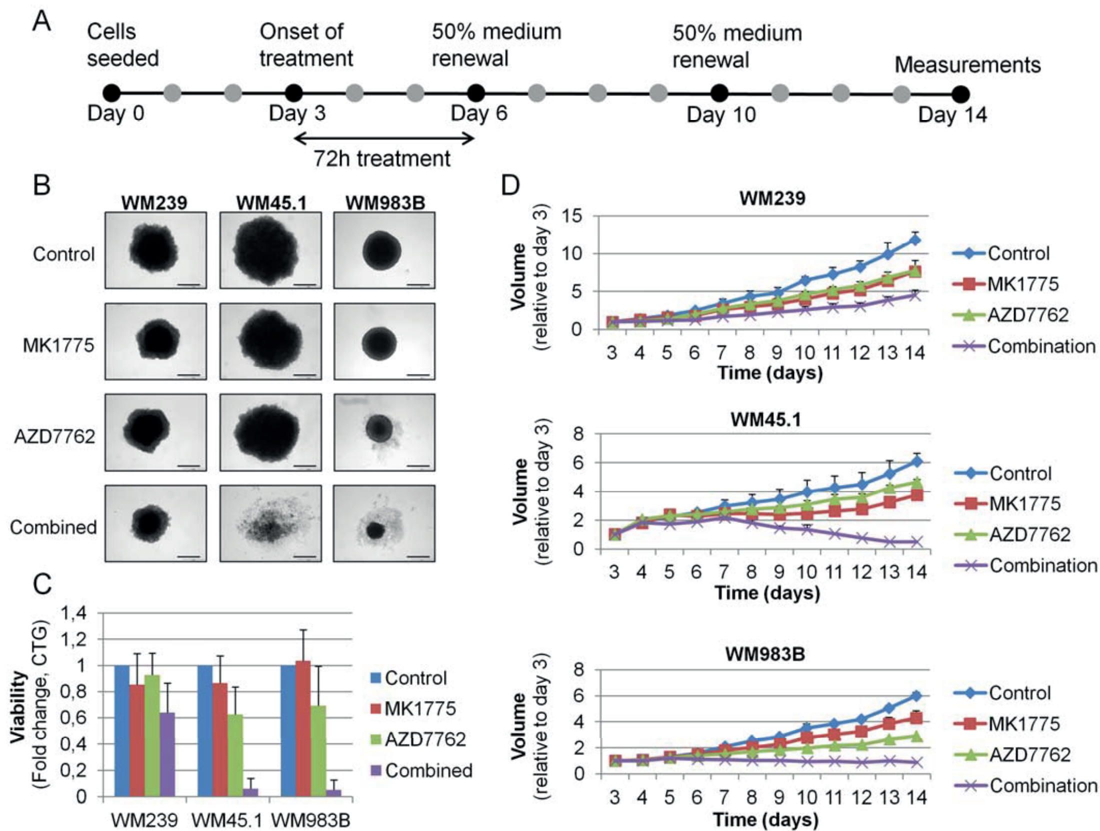


FIGURE 2

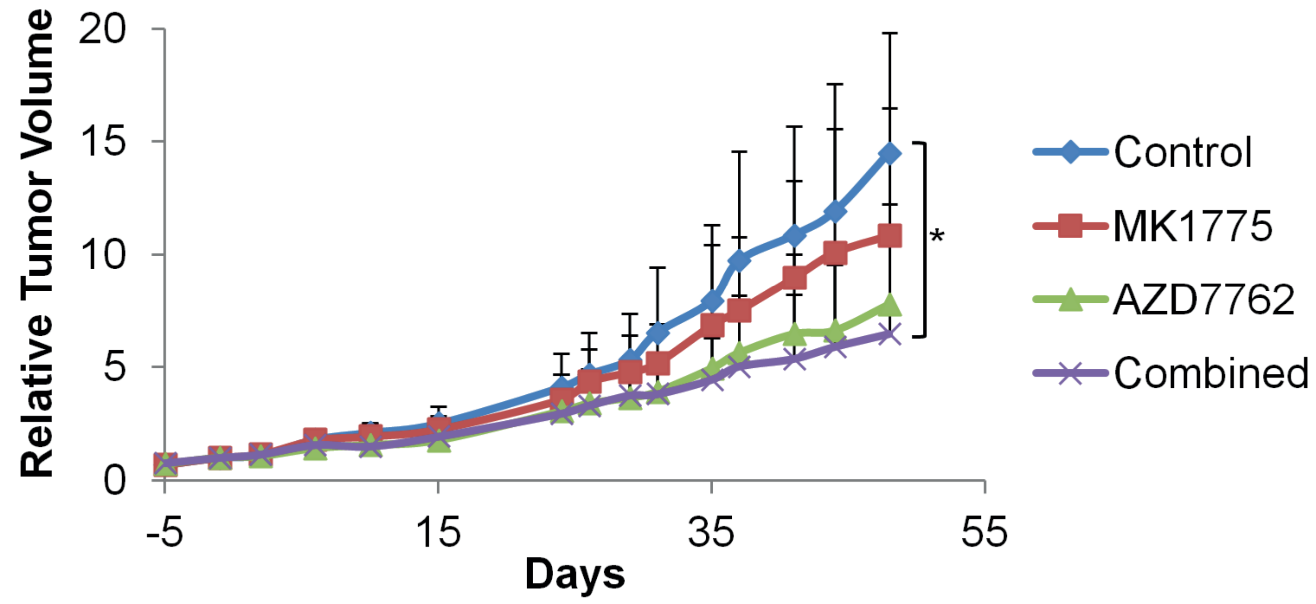
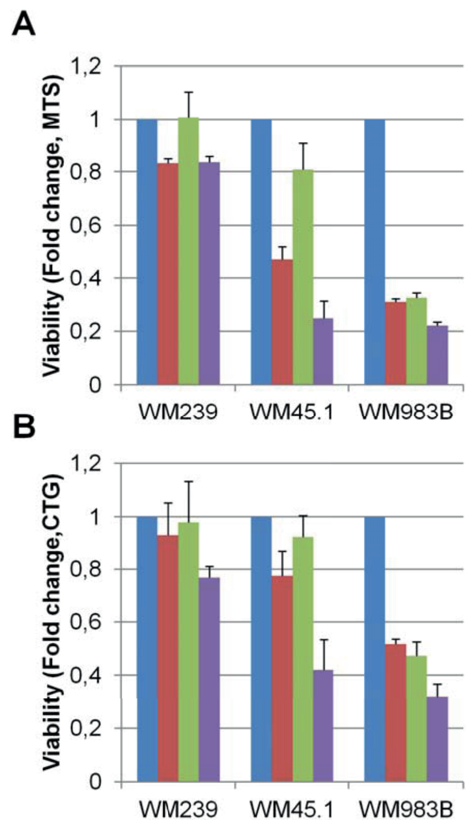
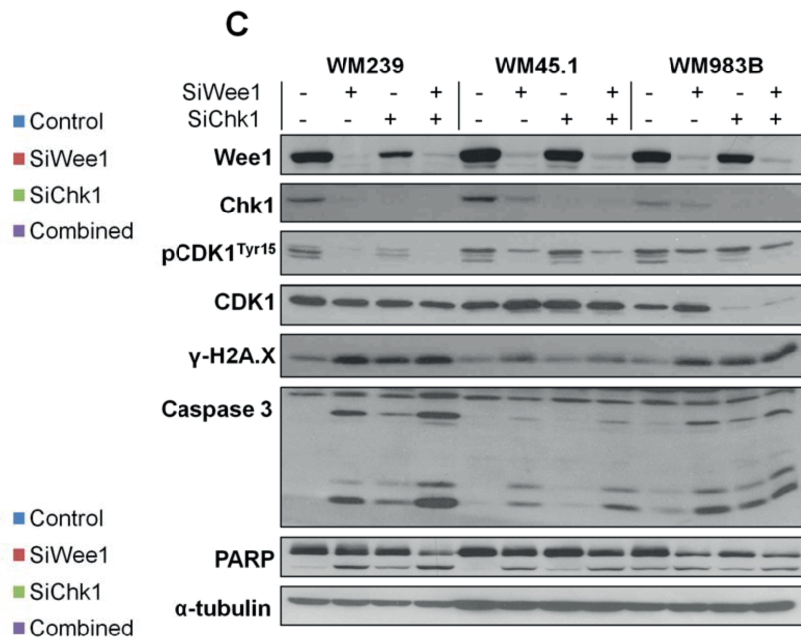


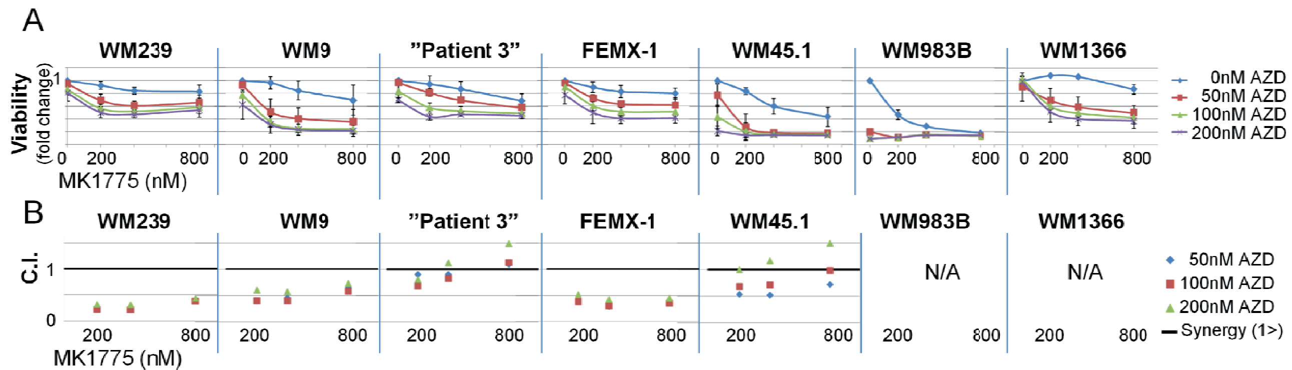
FIGURE 3

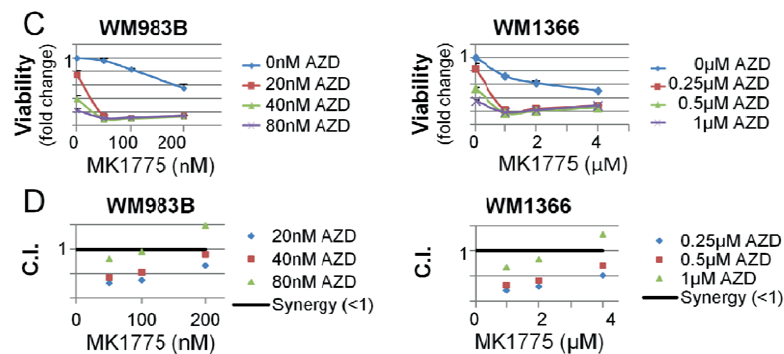
FIGURE 4



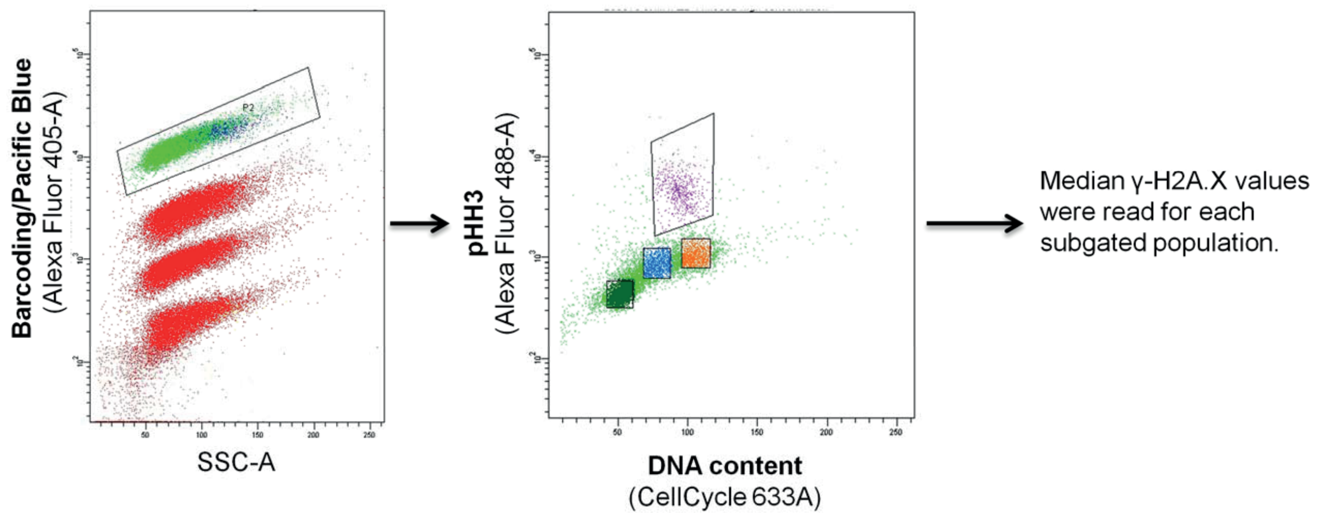


Suppl. FIGURE 1

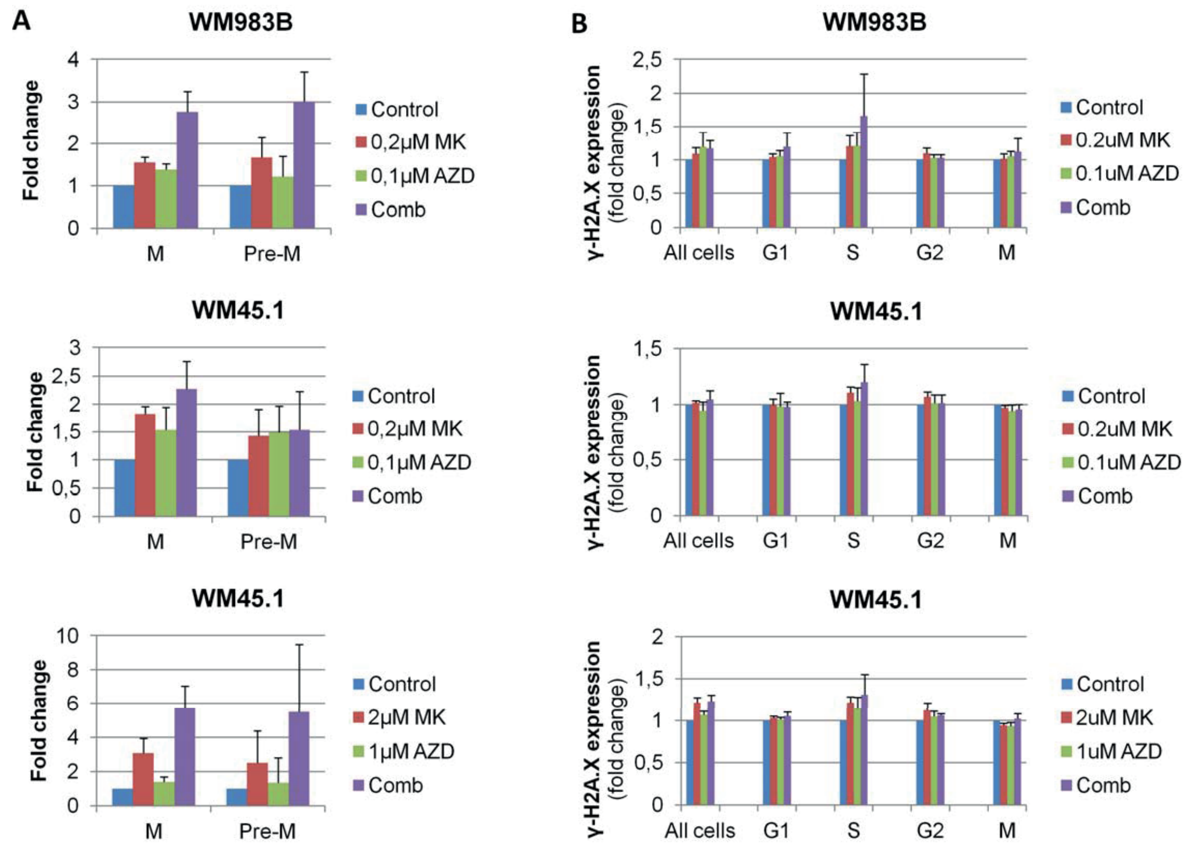




Suppl. FIGURE 2



Suppl. FIGURE 3



Suppl. FIGURE 4