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Inhibition of the kinase Wee1

-

Cytotoxic mechanisms and autoprotection by the tumor suppressor p53

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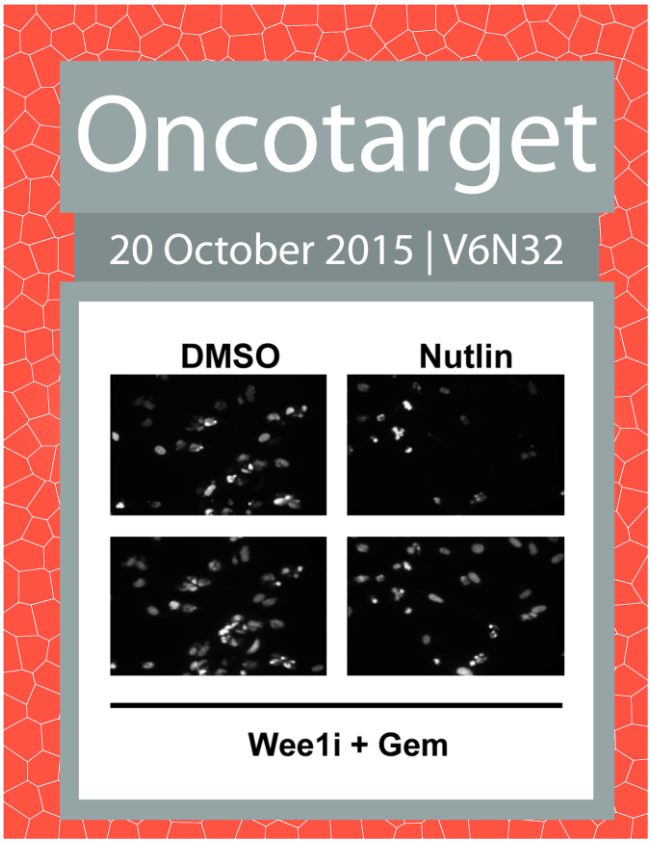
Abbreviations

°C	Degree Celsius
µl	Microliter
µM	Micromolar
ALL	Acute lymphoblastic leukemia
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
Bcl	B-cell lymphoma
BSA	Bovine serum albumin
CAK	CDK-activating kinase
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CML	Chronic myeloid leukemia
C-terminus	Carboxy terminus
dCK	deoxycytidine kinase
dFCTP	2',2'-difluorodeoxycytidine triphosphate
DDR	DNA damage response
DISC	Death-inducing signaling complex
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	deoxynucleoside triphosphate

dsDNA	Double stranded DNA
DSB	Double stranded DNA break
H2AX	Histone variant 2AX
H_2O	water
hNT	human nucleoside transporter
HR	homologous recombination
HRP	Horse Radish Peroxidase
IC value	Inhibitory Concentration value
kDa	Kilodalton
M	Molar
MDC1	mediator of DNA damage checkpoint 1
Mdm2	Mouse double minute 2
mg	milligram
min	minute
miRNA	micro RNA
ml	milliliter
mM	milimolar
MPF	mitosis promoting factor
MRN	MRE/Rad50/NBS1
mRNA	messenger RNA
NHEJ	non-homologous end joining
NER	nucleotide excision repair
PBS	phosphat buffered saline
PARP	Poly-ADP-Ribose-Polymerase
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction

PI	Propidium Iodide
Plk1	Polo-like kinase 1
qPCR	quantitative PCR
pRb	retinoblastoma protein
RNA	ribonucleic acid
RNF	ring finger protein
RPA	replication protein A
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
ssDNA	single stranded DNA
TBST	Tris buffered saline + Tween20
Tris	Trisamine
UV	Ultraviolet
V	Volt
Wee1i	Wee1 kinase inhibitor
γH2AX	Gamma-H2AX

Publications derived from this work:



Priority research paper, shared first author, Oncotarget cover story:

Li Y, Saini P, Sriraman A, Dobbelstein M (2015): Mdm2 inhibition confers protection of p53-proficient cells from the cytotoxic effects of Wee1 inhibitors. Oncotarget 6, 32339–32352 (cover page depicted above)

Priority research paper, co-corresponding author:

Sriraman A, Radovanovic M, Wienken M, Najafova Z, **Li Y,** Dobbelstein M (2016): Cooperation of Nutlin-3a and a Wip1 inhibitor to induce p53 activity. 2016 May 31;7(22):31623-38.

Research paper, co-author:

Saini P, **Li Y,** Dobbelstein M (2015b): Wee1 is required to sustain ATR/Chk1 signaling upon replicative stress. Oncotarget 6, 13072–13087

Editorial, co-author:

Saini P, **Li Y,** Dobbelstein M (2015a): Wee1 and Chk1 - crosstalk between key players in replicative stress. Genes Cancer 6, 182–183

Sriraman A, **Li Y,** Dobbelstein M (2016) Fortifying p53 - beyond Mdm2 inhibitors. Aging (Albany NY). 8(9):1836-1837.

Abstract

The combination of the Wee1 inhibitor MK-1775 and gemcitabine is highly efficient in killing cancer cells *in vitro* and in mouse xenograft experiments, but the complete molecular mechanism of this potent sensitizing effect remains unknown. We found that MK-1775 does not only block Wee1 activity in gemcitabine treated cells, but also reduces the activation of the ATR/Chk1 pathway in a Cyclin-dependent kinase 1 (Cdk1) dependent manner. These findings suggest that Wee1 inhibitors do not only interfere with cell cycle checkpoints to force cell cycle progression, but also to enhance replicative stress and intensify chemosensitivity towards nucleoside analogues through Chk1 inhibition and replicative stress, making them interesting therapeutic agent candidates for clinical oncology. However, considerable MK-1775 toxicities have been observed in preclinical as well as in clinical trials.

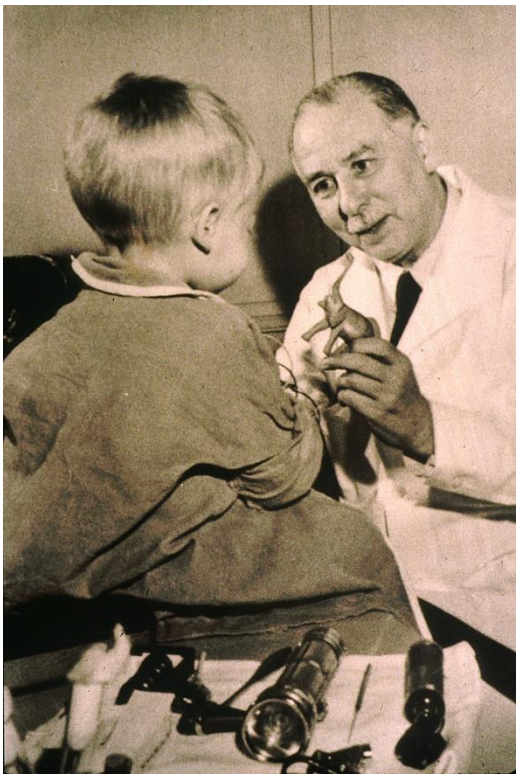
Over 50% of all cancers carry a mutation in the TP53 gene. Using the MDM2-antagonist Nutlin-3a, we provide a selective protection of p53-proficient cells against the cytotoxic effects of Wee1 inhibitors. Pretreatment of p53 wildtype cells with Nutlin-3a results in a transient cell cycle arrest, which effectively benefits cell survival upon subsequent treatment with the combination of the Wee1 inhibitor MK-1775 and gemcitabine. Nutlin-3a pretreatment reduced both the DNA damage response, as well as caspase activation in a p53-dependent manner. MDM2 antagonists might therefore selectively protect p53-proficient cells against the cytotoxic effects of Wee1 inhibitors, especially when combined with an S-phase specific drug, such as the nucleoside analogue gemcitabine. This approach might help to avoid toxic side effects of Wee1 inhibitors in anticipated clinical applications.

I Introduction

I.1 Cancer and the origins of chemotherapy

Cancer is a group of related diseases, in which cells undergo malignant transformation to promote uncontrolled cell growth and loss of differentiation. They all have a genetic cause of malignancy. Furthermore, some cancers will metastasize in later stages to evolve into systemic illnesses. If metastasis has not yet occurred, local tumors can be operated and/or irradiated. These procedures are very promising and can lead to complete remission of the patient. Unfortunately, a metastasizing tumor cannot be efficiently eradicated by local treatments. Until now, the only promising approach for late stage metastasized cancers is chemotherapy. Chemotherapy is applied orally or injected into the bloodstream or spinal fluid of the patient and acts on the entire human system. This way, even metastases too small to be detected by our current diagnostic tools can be targeted and destroyed.

The pioneer of cancer chemotherapy, Sidney Farber, has established the folic acid antagonist aminopterin as an intravenous chemotherapeutic drug to send young acute lymphoblastic leukemia (ALL) patients into temporal remission. This revolutionary approach, this systemic targetting on leukemia, a cancer of the blood, was the onset of modern chemotherapy. Farber's efforts to treat cancer were not limited to the application of aminopterin, he worked relentlessly to establish different chemotherapies and to develop a cure for cancer in general (Mukherjee 2011). Over the last century it has become clear, that cancers are as heterogeneous as their host patients, as not all chemotherapeutical drugs would work with the same efficiency on different patients with the same type of cancer. Overcoming this obstacle is the promise of personalized medicine, with more and more advanced technologies and computational power, we will soon be able to reveal the weaknesses of individual tumors in single patients at affordable expenses. To exploit these weaknesses, we also need to broaden the variety of combinatory chemotherapeutical drug regimen. Therefore, personalized medicine is an interdisciplinary approach: We need physicists and computer scientists to improve information technology, chemists to synthesize a broad variety of new substances, biologists to test these putative drugs in cell lines and animal models, and finally empower medical doctors to evaluate the information about the unique cancer of the individual patient to apply a personalized chemotherapeutic drug regimen to treat the tumor.



Farber's dream of finding cures through chemotherapy lives on, and the promise is renewed by the prospect of personalized medicine. We might not ever be able to cure all cancers, but we might take off the death sentence from a cancer diagnosis, as it has happened with once lethal diseases such as multiple sclerosis, diabetes and AIDS, and turn it into a chronic disease, with impaired life quality, but non-lethal, giving these patients decades more to live.

Figure I.1: Sidney Farber with a young patient in 1960 (wikipedia.com).

I.2 Cell cycle and molecular responses to DNA damage

The cell cycle is an important molecular machinery all eukaryotes share in common, this intricate system tightly regulates cell growth, DNA replication, mitosis, apoptosis and senescence; without it, multicellular life would be impossible. The necessity for a functional cell cycle in higher eukaryotes can be observed in the pathology of cancer: Uncontrolled cell divisions lead to tumor formation, metastasis and inevitably to the death of the organism. In this case, regulatory mechanisms governing the cell cycle have been lost or hijacked by the disease (Chow and Poon 2010). Untransformed cells are resistant to cancerogenesis through various mechanisms: One important feature is the ability to detect, measure and repair DNA damage (Hanahan and Weinberg 2011). This core machinery is encased within the cell cycle and tightly regulated by the so-called cell cycle checkpoints, which are governed by various important proteins, including the famous p53 protein, the guardian of the genome. p53 is of such importance, because it stands at the crossroads of a cells decision to survive or to undergo apoptosis. It gathers various cellular inputs to either promote cell cycle arrest and subsequent DNA damage repair, or to have the cell undergo programmed cell death (Bieging and Attardi 2012). Because of these features, it is not surprising that p53 is the most frequently mutated gene in human cancers, almost 50% of all cancers carry a TP53 mutation,

the protein has therefore established itself as the most investigated molecule in cancer research (Vogelstein et al. 2000).

I.2.1 Cell cycle regulation through Cyclins and Cdks

The tight regulation of the cell cycle is ensured by various Cyclins and Cyclin-dependent kinases (Cdks) (Bloom and Cross 2007). Cdks are the key regulators of the cell cycle, which are activated through hetero-dimerization with their corresponding Cyclins and subsequent phosphorylation by Cdk-activating kinases (Caks) (Malumbres and Barbacid 2009). Different Cyclin-Cdk complexes are specific to their respective cell cycle phase: In the G1 phase, in which the cell synthesizes molecules in preparation for the S-phase, Cyclin D forms complexes with Cdk4 and Cdk6. In the late G1-phase, Cyclin E complexes with Cdk2, this combination is called the S-phase promoting complex. The S-phase induced Cyclin A-Cdk2 complex arises during DNA replication and remains stable throughout the G2 phase until the cell enters mitosis. Finally, the Cyclin B-Cdk1 complex, historically called the mitosis promoting factor (MPF) is crucial for the G2/M transition (Malumbres and Barbacid 2009).

I.2.2 Activation of cell cycle checkpoints

The cell cycle needs to be tightly orchestrated to ensure the generation of two healthy daughter cells, for which cell size and DNA ploidy, to ensure a viable cell size and to prevent aneuploidy, are crucial parameters. Regarding this already intricate machinery alone, it is still insufficient to protect the genome from genotoxic stress, DNA damage and subsequent mutations. Therefore, evolution has developed an emergency control mechanism by introducing checkpoints to the cell cycle: DNA damage induces cell cycle arrests at the G1/S and G2/M checkpoints. Furthermore, the S-phase cell is also able to stop the cell cycle upon replicative stress, and induces the so-called intra-S checkpoint (Leemans et al. 2011).

Upon genotoxic stress, the G1/S checkpoint is activated and halts the cell cycle. This process is tightly regulated by p53 (Leemans et al. 2011). The canonical DNA damage kinases ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3-related) become active upon DNA damage and transduce their signal through the Checkpoint kinases 1 and 2 (Chk1 and Chk2) (Bouwman and Jonkers 2012). ATM and ATR induce the degradation of Cdc25A, an activating phosphatase of Cdks. Loss of Cdc25A inhibits DNA replication by inactivation of the Cyclin E/A-Cdk2 complex (Tse et al. 2007). ATM and ATR further activate p53 by phosphorylation at

Ser15 and Ser20, which then, as a tetrameric transcription factor, activates its target genes to promote cell cycle arrest, DNA repair and possibly apoptosis (Kastan and Bartek 2004). For the G1/S phase, the prominent p53 target gene p21 acts as a Cyclin E/A-Cdk2 and Cyclin D/Cdk4,6 inhibitor. Furthermore, p21 binds to PCNA, hampering with the DNA replication core machinery itself (Funk et al. 1997). p21, acting as powerful gatekeeper for S-phase entry, is therefore strongly prohibiting DNA replication progression by two distinct mechanisms.

Another important regulator of the G1/S transition is the RB/E2F1 complex. The retinoblastoma (RB) protein has been discovered to be an important tumor suppressor protein, loss of the RB protein leads to the malignant pathology of retinoblastoma, most frequently diagnosed in infants (Nevins 1992). The RB/E2F1 complex is regulated by phosphorylation through Cyclin D/Cdk4,6 and Cyclin E/Cdk2 complexes, a phosphorylated RB protein cannot bind to the E2F1 transcription factor, which then induces Cyclins E and A to promote entry into S-phase. Upon genotoxic stress, p53 activation and inhibition of the Cyclin/Cdk complexes through p21, RB protein is predominantly found in its dephosphorylated state, in which it binds and inactivates E2F1, prohibiting its transcriptional activity and thereby halting the G1/S cell cycle transition (Kastan and Bartek 2004). Therefore, the RB/E2F1 complex represents another switch-like regulatory mechanism for S-phase entry and progression, which also acts downstream of p21.

Within the S-phase exists another checkpoint protecting the replicating genome from genotoxic stress, the so-called intra-S-checkpoint. It is also activated through ATM and ATR signaling pathways, which lead to inhibition of Cdk1 and Cdk2 activity through p21 (Bartek and Lukas 2003). Upon activation of this checkpoint, the nucleus seizes origin of replication firing and activates DNA repair mechanism pathways (Kastan and Bartek 2004).

When DNA damage is present during the G2 phase, the cell triggers activation of the G2/M checkpoint. Upon its activation, both the ATR-Chk1 and the ATM-Chk2 axis target the mitosis promoting factor (MPF), i.e. Cyclin B/ Cdk1 (Kastan and Bartek 2004), the cell cycle will halt at the entry into mitosis. Three p53 target genes contribute to the inhibition of the MPF, which are GADD45, p21 and 14-3-3σ (Taylor and Stark 2001). p21 inactivates Cdk1 directly, whereas 14-3-3σ traps Cdk1 in the cytoplasm, preventing its mitotic inducing activity. GADD45 interferes with Cyclin B / Cdk1 complex formation, thus decreasing Cdk1 activity (Zhan et al. 1999). All three factors thus hamper with Cdk1 function, preventing the cells entrance into mitosis.

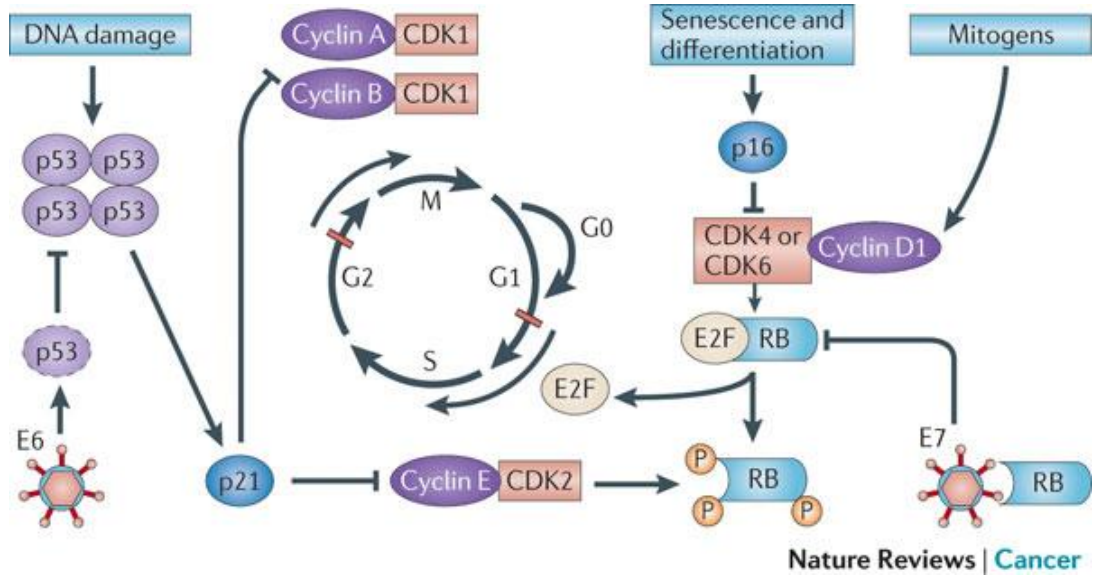


Figure I.2: Cell cycle regulation by different cell cycle checkpoint control pathways.

When DNA damage occurs during the G1 phase of the cell cycle, it activates the G1/S checkpoint, which is controlled by both the p53 and the RB/E2F pathways. RB binds E2F, preventing its S-phase inducing function and the p53 downstream effector protein p21 inhibits the Cyclin E / Cdk2 complex which promotes replication progression by phosphorylating and inhibiting RB function. Furthermore, p21 it is able to inhibit the G2/M checkpoint upon DNA damage in the G2 phase by counteracting the mitosis promoting Cyclin A and B / Cdk1 complexes (Image taken from Leemans et al. 2011).

I.2.3 Replicative stress and DNA damage response pathways

The genome is most vulnerable during the S phase of the cell cycle. During unwinding and replication of the DNA, many errors and chemical modifications can occur to the macromolecule, these events can be summarized and described as DNA damage. DNA damage endogenously occurs in the cell at high rates, mostly induced by its oxidative metabolism, but it can also be induced exogenously by chemical or irradiation stressors (Kastan and Bartek 2004). The ability of the cell to repair these damaged DNA sites is crucial for its survival. Accumulated DNA damage can lead to mutations and subsequent cancerogenesis, DNA repair pathway genes are thus often mutated in transformed cells. Paradoxically, oncologists utilize DNA damage through chemo- and radiotherapy to kill cancer cells, but at the same time they might lay the base for a secondary tumor to occur (Boffetta und Kaldor 1994; Ng und Shuryak 2014). It is therefore crucial to gain an in-depth understanding of DNA damage and the cells response towards it. Canonically, DNA damage is detected through two hallmark kinases of the PI3K (phosphatidylinositol 3-kinase related kinase) family, which are ATR and ATM, the two first line DNA damage kinases (Giglia-Mari et al. 2011).

I.2.3.1 The ATR-Chk1 pathway responds to single-stranded DNA breaks (ssDNA breaks)

Single-stranded DNA breaks (ssDNA breaks) in the cell can arise through different mechanisms: Genotoxic stress in S-phase leads to replication fork stalling, the replication fork stops, but the helicase continues to unwind the double-stranded DNA (dsDNA), directly exposing ssDNA (Kastan and Bartek 2004). Upon ssDNA formation, the lesion site is immediately covered by replication protein A (RPA), which then recruits the ATR - ATR interacting protein (ATRIP) complex (Zou and Elledge 2003). ATR transmits its signal through phosphorylation of various substrates, most importantly Chk1 (Liu et al. 2000). Chk1 is able to halt the cell cycle through various functions: Chk1 mediated phosphorylation leads to proteasomal degradation of the Cdc25A and Cdc25B phosphatases, which remove inhibitory phosphorylations from Cdks. Chk1 is therefore able to stop cell cycle progression at any given cell cycle checkpoint by functionally inhibiting Cdks (Chen and Poon 2008). Furthermore, Chk1 activates Wee1, a kinase that introduces inhibitory phosphorylations to Cdk1 at Tyr15 and Thr14, which halt the cell cycle at the G2/M-phase checkpoint (Smith et al. 2010). The inhibitory phosphorylation at Tyr15 can be removed by Cdc25C, thus activating Cdk1. Chk1

can phosphorylate Cdc25C at Ser216, the phosphorylated form is bound by 14-3-3 proteins and therefore removed from the active pool and the G2/M cell cycle checkpoint stays activated due to impaired Cdk1 function (Peng et al. 1997). Chk1 signaling is therefore able to inhibit Cdks and thus cell cycle progression through distinct mechanisms.

I.2.3.2 The ATM-Chk2 pathway responds to DSB

If stalled replication forks persists, dsDNA breaks form upon disintegration of the replication complex. This process signals to inactive ATM dimers, which auto-phosphorylate at Ser1981 and dissociate to active ATM monomers (Bakkenist and Kastan 2003). ATM is then recruited to the DNA damage site by the MRE11/RAD50/NBS1 (MRN) complex (Lee and Paull 2007). At the site of DNA damage, ATM activates its target protein checkpoint kinase 2 (Chk2) via phosphorylation at Thr68 (Buscemi et al. 2004). Like Chk1, Chk2 down-regulates Cdc25 protein levels and promotes cell cycle arrest at various cell cycle checkpoints (Lee and Paull 2007). Furthermore, both ATM and Chk2 phosphorylate and activate the transcription factor p53. In addition, ATM hampers with the MDM2/p53 auto-regulatory negative feedback loop by phosphorylating MDM2 at Ser395 (Buscemi et al. 2004). Ser395 lies within the N-terminus of MDM2, its phosphorylation impairs the ubiquitination of and subsequent degradation of p53. Most interestingly, phosphorylation at Ser395 also enhances ubiquitination and subsequent protein degradation of MDM2 itself (Valentine et al. 2011). Through elevated levels of activated p53 by decreasing MDM2 function, the ATM-Chk2 axis regulates the cell fate upon genotoxic stress.

Nucleosomes are composed of histone octamers, in a single octamer one can find two copies of histone H2A. In about every fifth nucleosome, H2A is replaced by its isoform H2AX, which is different in its biological features (Redon et al. 2002). Upon activation of the DNA damage signaling pathways, H2AX is phosphorylated at Ser139 by the ATM kinase, which is then called γH2AX (Huang et al. 2004). The scaffold of γH2AX, ATM and mediator of DNA damage checkpoint 1 (MDC1), which is also phosphorylated by ATM (Lou et al. 2006), is crucial in supporting the spread of this DNA damage signal. Subsequently, the ring finger proteins 8 and 168 (RNF8 and RNF168) E3 ubiquitin ligases add poly-ubiquitin residues to γH2AX, creating a scaffold for further downstream DNA double strand repair mechanism such as non-homologous end joining (NHEJ) or homologous recombination (HR) (reviewed in van Attikum and Gasser 2009).

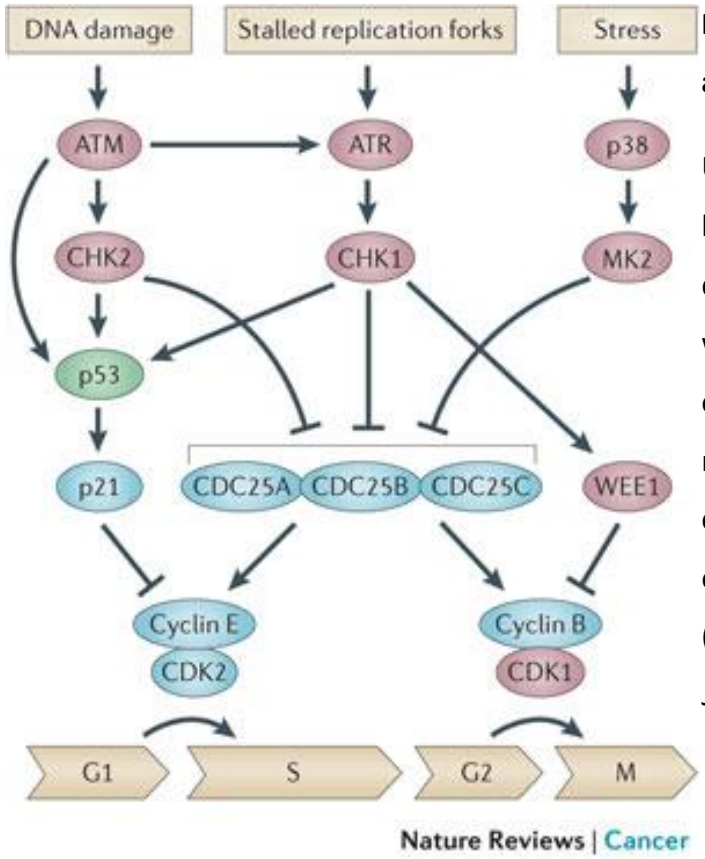


Figure I.3: Cell cycle checkpoints are activated by Chk1 and Chk2 kinases.

Upon different stressors, the apical kinases ATM and ATR activate their downstream kinases Chk2 and Chk1, which either act via the p53-p21 axis or through CDC25 phosphatase mediated inhibition of Cyclin/Cdk complexes to halt the cell cycle at either the G1/S or G2/M transition (Image taken from Bouwman and Jonkers 2012).

I.2.4 p53 – The guardian of the genome

Since its discovery in 1979, p53 has established itself as the most studied protein in the field of cancer biology. The interest in p53 is based on its important function as a molecular switch for a cell to either live or die, which makes p53 the most important tumor suppressor gene known. This hypothesis is further supported by the observation, that 50% of all cancers will carry a mutation in the TP53 locus (Vogelstein et al. 2000). p53 accumulates upon DNA damage and halts the cell cycle, it then evaluates to either repair the damage or to induce programmed cell death, so-called apoptosis. All these molecular mechanisms are governed by p53 through its transcriptional activation of its plethora of target genes.

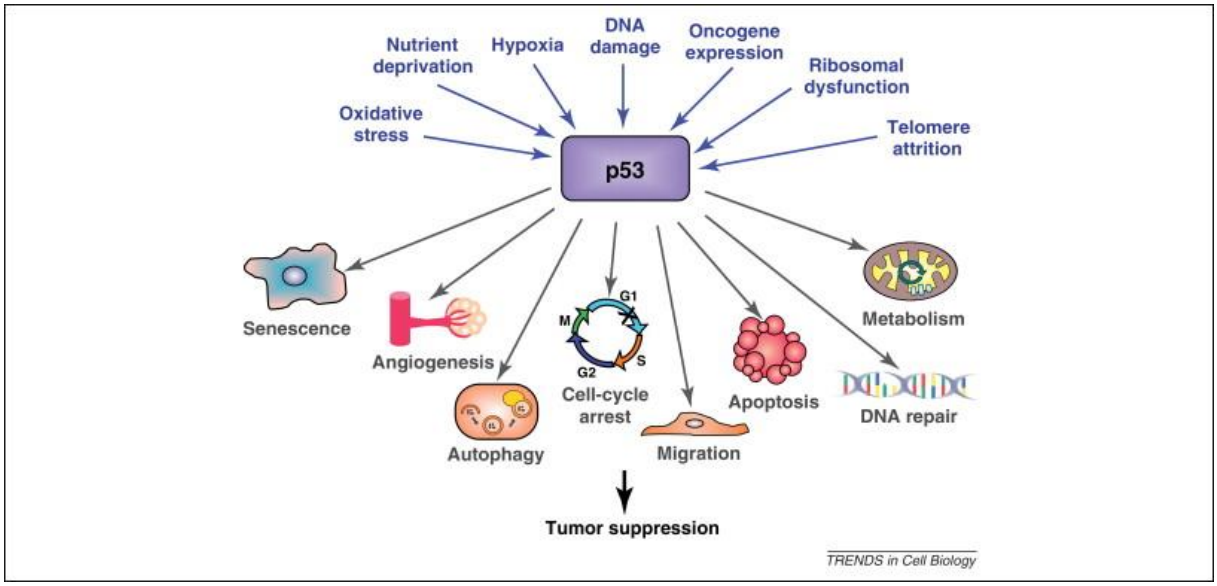


Figure I.4: p53 at the hub of cellular stress responses.

The p53 pathway is activated upon various cellular stressors and has a wide range on cellular responses to these different types of stresses. The triggered responses range from cell protective, such as DNA repair, to cell destructive reactions, such as apoptosis. Taken together, the p53 pathway is a powerful molecular machinery, which can decide the fate of a cell upon external and internal stressors (Image taken from Bieging and Attardi 2012).

I.2.4.1 p53 stabilization, activation and transcriptional activity

Different stressful conditions lead to various post-translational modifications of p53, including addition or removal of phosphate, acetyl, ubiquitin and sumo residues (Meek 1999). Upon DNA damage, p53 is activated and stabilized by the canonical DNA damage response kinases ATM, ATR, Chk1 and Chk2, through phosphorylation at Ser15 and Ser20 (Vogelstein et al. 2000). Stabilization occurs due to the disruption of the p53/MDM2 interaction site through ATM and ATR mediated phosphorylation at Ser15, which lies within the binding pocket of this protein-protein interaction (Milczarek et al. 1997). Chk1 and Chk2 promote tetramerization of the protein via phosphorylation at Ser20, thus enhancing the transcriptional activity of p53 (Meek 1999). Once in a homo-tetrameric complex, p53 activates a multitude of target genes, among the most intensively investigated are its negative regulator MDM2 itself, GADD45, the infamous Cdk inhibitor p21 and the pro-apoptotic bax protein (Tokino and Nakamura 2000). By transcriptionally activating numerous cellular functions, including cell-cycle arrest, senescence and apoptosis, the tumor suppressor p53 is thus able to decide the fate of a cell (Bieging and Attardi 2012).

I.2.4.2 The MDM2-p53 auto-regulatory negative feedback loop

p53 levels within a cell are mainly regulated by MDM2 mediated proteasomal degradation. MDM2 is a RING E3 ubiquitin ligase, which binds p53 and subsequently adds poly-ubiquitin chains to its target protein. Both the binding of MDM2 to the p53 N-terminal transactivation domain and the proteasomal degradation of p53 protein diminish its transcription factor activity and cellular functions (Michael and Oren 2003). p53 induces MDM2 mRNA transcription, this negative feedback loop therefore tightly controls p53 levels within a healthy cell. Upon genotoxic stress, the MDM2-p53 interaction is disrupted by DNA damage kinase mediated phosphorylation of p53 at Ser15 (Shieh et al. 1997). Furthermore, ATM phosphorylates MDM2 within the RING domain at Ser395, adding to the steric hindrance of the MDM2/p53 protein-protein interaction (Valentine et al. 2011).

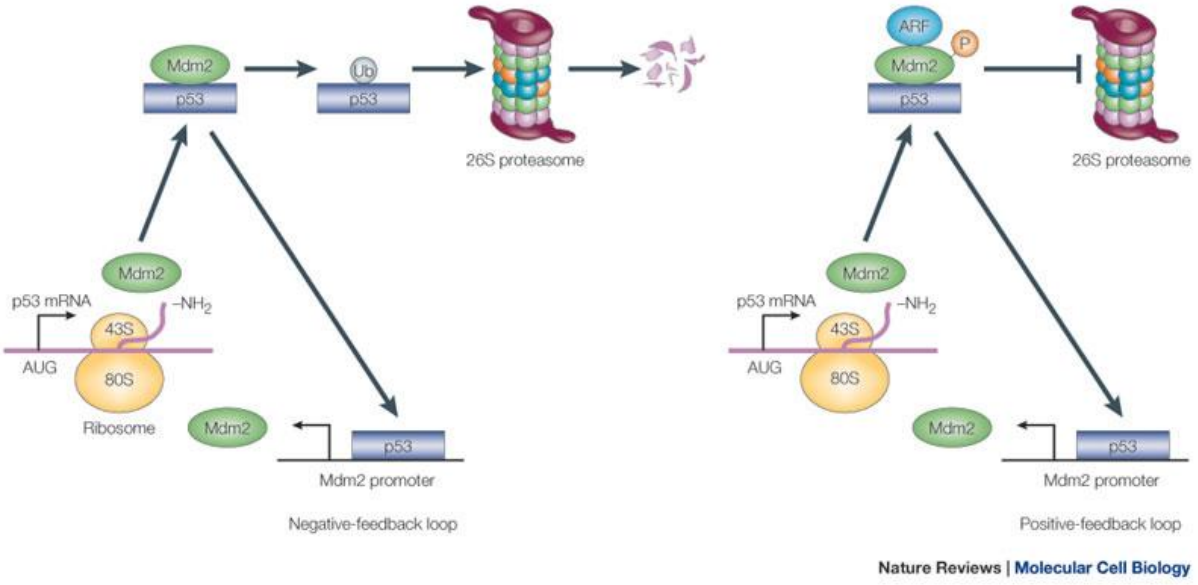


Figure I.5: p53-MDM2 feedback loops.

Two feedback loops can be distinguished within the p53-MDM2 interaction: The negative feedback loop describes the proteasomal degradation of p53 through MDM2 mediated protein poly-ubiquitination, whereas the positive feedback loop is created via p53 mediated transcriptional induction of MDM2 mRNA and subsequent high intracellular levels of MDM2 protein (Image take from Fahraeus 2005).

I.2.5 Extrinsic and intrinsic induction of apoptosis

Another important step for the evolution of multicellular organisms is the possibility to sacrifice a cell for the good of a genetically identical cellular population. This intrinsic removal of old or damaged cells is called programmed cell death or apoptosis (Elmore 2007). Since resistance to pro-apoptotic signals can promote the formation of cancers, this process is crucial for aging and survival of higher organisms.

Upon extensive and irreparable amounts of cellular stress, tumor suppressor proteins, such as p53, pressurize the cell to undergo programmed cell death, so-called apoptosis. In contrast to necrosis, in which catastrophic cell disintegration leads to cell membrane rupture and cytoplasm spilling into the extracellular space, triggering an inflammatory response, apoptosis itself is a highly regulated and organized, non-inflammatory process. The apoptotic cell detaches from its surrounding environment, all macromolecules are internally digested into fragments and the cell is formatted into multiple vesicles, called apoptotic bodies, which

are subsequently taken up and digested by macrophages (Elmore 2007). Apoptosis is therefore a silent way to remove dysfunctional cells from a homeostatic cell population.

Intracellular signaling of apoptosis is governed by the class of caspase proteins (cysteine-dependent aspartate-directed proteases). Caspases are activated from their inactive precursor form, so-called procaspases, by catalytic cleavage from another activated caspase protein (Nunez et al. 1998). Initiator caspases are part of activating protein complexes, for instance, caspase 9 is part of the apoptosome complex, whereas caspases 8 and 10 are essential for the function of the death-inducing signaling complex (DISC) (Nunez et al. 1998). One important function of downstream effector caspases, such as caspases3, 6 and 7, is the activation of caspase activated DNAses (CADs), which fragment genomic DNA by cutting predominatly between the nucleosomes, creating the apoptotic phenomenon of DNA laddering when run on an agarose gel (Nunez et al. 1998).

Intrinsic activation of apoptosis is triggered upon extensive cellular stress, such as massive DNA damage. The main trigger for intrinsic apoptosis is the release of cytochrome c from the mitochondria into the cytoplasm forming the caspase activating apoptosome (Ashkenazi 2008). Cytochrome c is released due to increased porosity of the mitochondrial membrane, the stability of the latter is determined by a delicate balance of pro- and anti-apoptotic proteins at the mitochondrial surface, which is critically influenced by p53 transcriptional activity. Both pro- and anti-apoptotic proteins belong to the Bcl-2 protein family, they are further classified into three subgroups: Anti-apoptotic, such as Bcl-2 and Bcl-XL, pro-apoptotic, such as Bax and Bak, and pro-apoptotic activating proteins of the BH-3 family, such as Bid, Bad, Puma and Noxa (Tait and Green 2010). Anti-apoptotic proteins are outer mitochondrial membrane proteins, whereas most pro-apoptotic proteins can be found within the cytosol (Hardwick and Soane 2013). Upon activation of intrinsic apoptotic signaling, the pro-apoptotic proteins Bax and Bak undergo a conformational change and integrate into the outer mitochondrial membrane. Subsequent oligomerization of the proteins form pores into the mitochondria, releasing cytochrome c into the cytosol (Tait and Green 2010). Cytosolic cytochrome c binds Apaf-1 (apoptotic protease activating factor 1), leading to the formation of the so-called apoptosome, which, through activation of caspase 9, transduces its signal to caspase 3, leading into a common output pathway with the extrinsically activated pathway of apoptosis. Importantly, caspase 3 also cleaves Poly-ADP-

Ribose-Polymerase (PARP), which is frequently used as a molecular marker for apoptosis (Boulares et al. 1999).

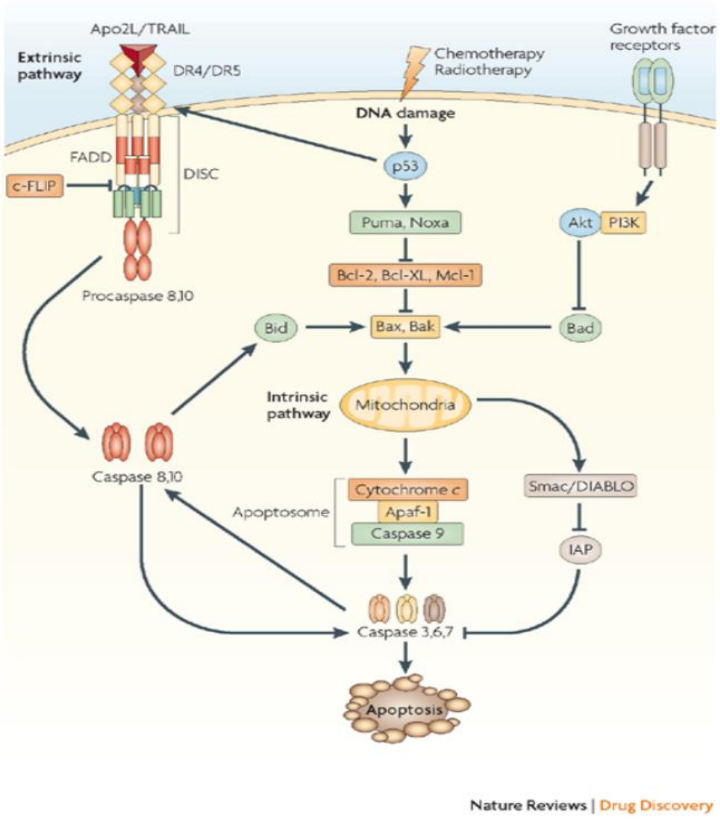


Figure I.6: Apoptosis can be induced through intrinsic as well as extrinsic cues. Upon DNA damage, p53 induces pro-apoptotic proteins of the BH-3 family, e.g. Puma and Noxa, these activate the pro-apoptotic proteins Bax and Bak by inhibiting anti-apoptotic proteins, such as Bcl-2 and Bcl-XL. Bax and Bak trigger the release of cytochrome c from the mitochondria, which contributes to the formation of the apoptosome. This complex activates the effector caspases 3, 6, and 7 via the initiator caspase 9, this activation effectively induces apoptosis in the cell. The effector caspases can also be activated by extracellular cues (Image taken from Ashkenazi 2008).

I.3 Chemotherapeutic drugs

Today, there are numerous classes of classical chemotherapeutics, which resemble cellular toxins, such as alkylating agents, antimetabolites, topoisomerase and mitosis inhibitors, platinum compounds and others (Espinosa et al. 2003). More recently, with an increased understanding of molecular cancer biology, new drugs targeting single proteins, so-called small molecule inhibitors in targeted therapies, have gotten into the focus of translational research approaches and clinical trials (Wu et al. 2015). Amongst the small molecule inhibitors, Imatinib (Gleevec®) has risen to fame for being a single drug to send a large proportion of chronic myeloid leukemia (CML) patients into complete remission (Hochhaus 2004; Roskoski 2015). The “magic bullet” is no longer fiction, but has become reality, at least in a small subset of human malignancies.

In this study, we have investigated the pharmacological functions and combinatory effects of the small molecule Wee1 kinase inhibitor MK-1775, the small molecule MDM2 inhibitor Nutlin-3a and the classical nucleoside analogue gemcitabine in human cancer cell lines.

I.3.1 Nucleoside analogues: Gemcitabine

Nucleoside analogues are a group of antimetabolites, which interfere with normal DNA and RNA synthesis. They are most effective in rapidly dividing cells, as these need to replicate their DNA at high rates and therefore have a high turnover of nucleotides. Their applications in cancer medicine are various, as they are active in solid tumors, metastases and hematological malignancies (Jordheim et al. 2006).

In our study, we have further investigated the classical nucleoside analogue gemcitabine, a first line drug for advanced ovarian and pancreatic cancers (Lorusso et al. 2006; Shore et al. 2003). Gemcitabine is a deoxycytidine/pyrimidine analogue, the hydrogen atoms at the 2’-carbon are substituted by two fluorine residues. After application, the prodrug gemcitabine is taken up into the cell via human nucleoside transporters (hNTs) and is then further phosphorylated by deoxycytidine kinase (dCK) to its monophosphate and subsequently into its main active triphosphate metabolite 2',2'-difluorodeoxycytidine triphosphate (dFdCTP), which is either incorporated into the DNA directly, or indirectly inhibits DNA synthesis through inhibition of ribonucleotide reductase, the rate limiting step of DNA synthesis (Veltkamp et al. 2008). Through its direct and indirect action on DNA synthesis and the

pharmacological stability of its active form, gemcitabine is a powerful drug capable of dealing collateral damage to dividing cells (Gesto et al. 2012). In more detail, gemcitabine is an efficient DNA replication specific drug, it depletes the deoxynucleoside triphosphate (dNTP) pool and stalls replication forks through steric hindrance during S-phase, creating massive amounts of DNA damage (Dobbelstein and Sorensen 2015). Such DNA damage during DNA replication is called replicative stress, which, through genome instability and mutations, has been named one of the new hallmarks of cancer (Hanahan and Weinberg 2011). By its actions, gemcitabine powerfully activates the G1/S phase checkpoint through its impact on DNA replication.

Gemcitabine is used as a first line drug in the very malignant pancreatic cancer (Burris et al. 1997; Moore et al. 2003). Nonetheless, medium survival rates remain low, as pancreatic tumors often develop resistance against gemcitabine, such as elimination of the drug from the cell through the human Nucleoside-Transporter 1 (hNT1) (Giovannetti et al. 2006) or increased nucleoside metabolism through upregulation of deoxycytidine kinase and ribonucleoside reductases M1 and M2 (Nakano et al. 2007). Therefore, chemo-sensitization of pancreatic cancer cells to gemcitabine through combinatory treatments is of great medical interest and clinical importance.

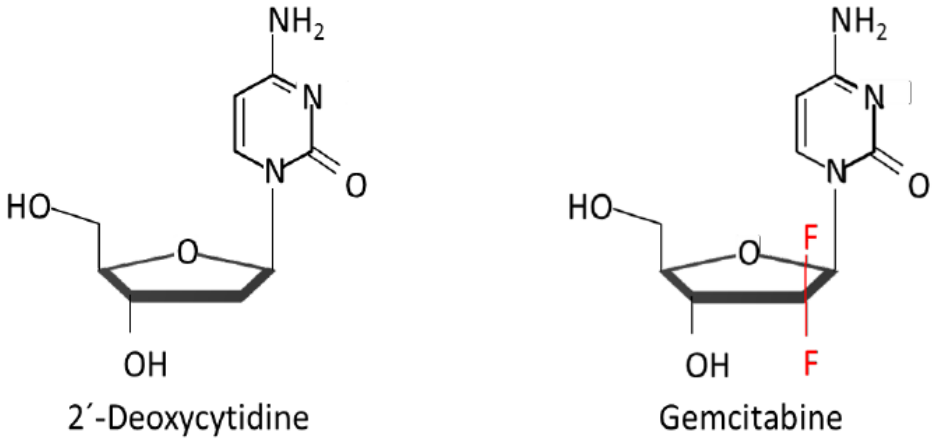


Figure I.7: Structures of deoxycytidine and gemcitabine.

Gemcitabine is a deoxycytidine/pyrimidine analogue, the hydrogen atoms at the 2'-carbon are substituted by two fluorine residues (modified from Ewald et al. 2008).

I.3.2 Small molecule inhibitors

Small molecule inhibitors are low molecular weight compounds, which inhibit a specific target protein, their application has therefore been coined targeted therapy. Some small molecule inhibitors prolong patient survival just for weeks or months longer, rendering them as ineffective in clinical trials, other compounds would send patients into stable complete remission, e.g. Imatinib (Roskoski 2015). In our study we have further investigated the Wee1 kinase inhibitor MK-1775 and the MDM2 inhibitor Nutlin-3a.

I.3.2.1 Wee1 kinase inhibitor (MK-1775)

The Wee1 kinase is an important regulator of the G2/M transition, this serine / threonine / tyrosine kinase adds inhibitory phosphorylations on Cdk1 at T14 and Y15 and thereby inhibits entry into mitosis (Watanabe et al. 1995). Wee1 protein levels and activity increase during S and G2 phase, peaking at the G2/M transition. Its activity decreases during M phase, where the protein gets hyper-phosphorylated by Cdk1 and Plk1 at Ser123 and Ser53, respectively, and is further subjected to proteolytic degradation through the E3 ubiquitin ligase SCFβ-TrCP1/2 (Watanabe et al. 1995; Watanabe et al. 2004; Ovejero et al. 2012). Inhibition of Wee1 and therefore uncontrolled Cdk1 activity forces S-phase-arrested cells directly into mitosis without completing DNA synthesis, resulting in cell death induced by mitotic catastrophe (Aarts et al. 2012). Furthermore, knockdown of the Wee1 kinase has been shown to stall DNA replication and to generate DNA damage, this is due to activation of the heterodimeric Mus81-Eme1 structure-specific endonuclease, which is capable of generating DSBs (Dominguez-Kelly et al. 2011).

MK-1775, a Wee1 small molecule inhibitor, has been found to sensitize cancer cells to a variety of DNA-damaging agents, including 5-fluorouracil (Hirai et al. 2010), gemcitabine and platinum based agents (Hirai et al. 2009), as Wee1 inhibition forces premature entry into mitosis upon DNA damaging agent induced cell cycle arrest. A xenograft experimental series has suggested a synergistic effect between MK-1775 and gemcitabine (Rajeshkumar et al. 2011). In this study, we have made an effort to describe this synergism mechanistically. Furthermore, although promising, MK-1775 has not achieved FDA approval due to enhanced cytotoxicity, such as myelosuppression and tachyarrythmia, in clinical trials (Do et al. 2015). A possibility to counter this toxicity could be cytoprotection of untransformed and p53

proficient cells through activation of cell cycle checkpoints. Therefore, we have investigated this hypothesis by co-treatment with the MDM2 small molecule inhibitor Nutlin-3a.

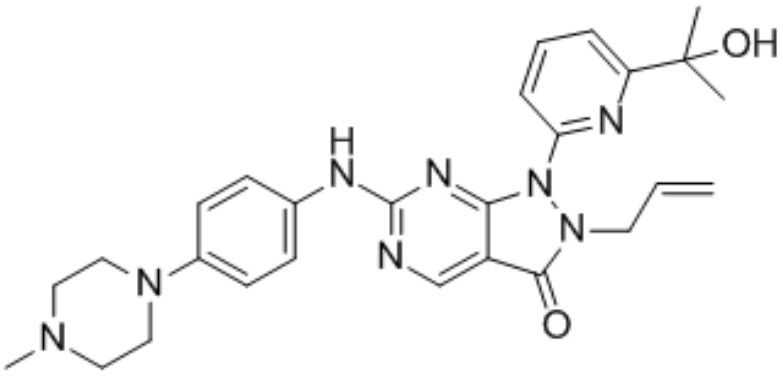


Figure I.8: MK1775 (MK-1775 medchemexpress.com)

I.3.2.2 The Mdm2 antagonist Nutlin-3a

Nutlin-3a is a small molecule inhibitor against the ubiquitin ligase MDM2, which is the main antagonist of p53 (Wade et al. 2013). Upon inhibition of MDM2, p53 accumulates and subsequently upregulates its target genes, such as p21 and MDM2 itself in a feedback loop fashion, leading to cell cycle arrest and possibly apoptosis (Khoo et al. 2014; Vassilev et al. 2004). Nutlin-3a acts in a non-genotoxic fashion (Miyachi et al. 2009) and stabilizes wildtype, but not mutant p53. It can therefore be utilized to protect untransformed cells from chemotherapeutics, such as mitosis active drugs (e.g. paclitaxel) and S phase active drugs (e.g. gemcitabine) (Carvajal et al. 2005; Kranz and Dobbelstein 2006). This observation might be of importance as approximately 50% of all tumors acquire a p53 functional deficiency during their malignant transformation (Vogelstein et al. 2000). Exploiting these genetic differences between malignant and untransformed cells might be a promising approach for clinical cancer research.

Nutlin-3a stabilizes p53 in a non-genotoxic fashion, as post-translational modifications specific to genotoxic stress do not appear on Nutlin-3a stabilized p53 (Shen and Maki 2011). This sounds like a good trait for a chemotherapeutic agent, but unfortunately, Nutlin-3a has been proven a weak drug in clinical trials, barely efficient against the rare tumor class of liposarcomas (Ray-Coquard et al. 2012). Prolonged treatment causes a prolonged cell cycle arrest, which is mostly reversible once the drug gets discontinued, further generating populations of resistant cells (Huang et al. 2009). Nutlin-3a has disappointed as the killer it

was designed to be, but its cellular protective function through p53 might be utilized for the concept of cyclotherapy (Blagosklonny und Pardee 2001).

We have exploited the possibility of cytoprotection through Nutlin-3a stabilized p53 against the potent trial combination regimen of gemcitabine and the small molecule Wee1 kinase inhibitor MK-1775.

I.4 Scope of the thesis

The aims of this study were the characterization of the mechanism behind the synergism of the chemotherapeutical combination of Wee1 kinase inhibition and gemcitabine treatment. Furthermore, we wanted to demonstrate a cytoprotective effect by activating p53 through treatment with Nutlin-3a against the potent combination of Wee1 inhibitor and gemcitabine. We wish to enforce the concept of cyclotherapy, giving the possibility of increasing chemotherapeutic drug concentrations to target malignant cells selectively with small molecule inhibitors and at the same time protect p53 untransformed cells pharmacologically from side effects. As the main difficulty of oncology is the specific targeting of the cancer, whilst avoiding collateral damage to normal cells, this concept of non-genotoxic chemical cytoprotection might help to distinguish these two cell populations within one cancer patient. This concept of cyclotherapy using a small molecule inhibitor might eventually be evaluated in a clinical trial.

II Materials and Methods

II.1 Materials

II.1.1 Technical devices

Table II.1.1 Technical Devices

Device	Company
Blotting chamber	Biozym, Hessisch Oldendorf, Germany
Centrifuge *5415R*	Eppendorf, Hamburg, Germany
Centrifuge *5810R*	Eppendorf
Chemiluminescence imager *Chemocam HR 16 3200*	Intas Science Imaging Instruments, Göttingen, Germany
Cytometer *Celigo*	Cyntellect, San Diego, CA, US
Electrophoresis system, for SDS-PAGE	Amersham Biosciences, GE Healthcare, UK
FACS machine *Guava PCA-96 Base*	Millipore, Merck, Darmstadt, Germany
Freezer -20°C	Liebherr, Bulle, Switzerland
Freezer -80°C	Heraeus, Thermo Scientific, MA, US
Heating Block	Grant Instruments, Hillsborough, NJ, US
Incubator for cell culture *Hera Cell 150*	Heraeus, Thermo Scientific, MA, US
Laminar flow cabinet *Hera Safe*	Heraeus, Thermo Scientific
Luminometer DLReady™Centro LB 960	Berthold, Bad Wildbad, Germany
Magnetic stirrer *MR Hei-Standard*	Heidolph, Schwabach, Germany
Mini Centrifuge *MCF-2360*	LMS, Tokyo, Japan
Multichannel Pipette *Transferpette S-8*	BrandTech Scientific, CT, US
pH-meter *WTW-720*	WTW, Weilheim, Germany
Pipets Eppendorf Research Series 2100	Eppendorf
Refrigerator 4°C	Liebherr
Roller RM5 V-30	CAT, Staufen, Germany

Scales *Acculab ALC-6100.1*	Sartorius, Göttingen, Germany
Scanner *CanoScan 8600F*	Canon, Tokyo, Japan
Shaker *PROMAX 2020*	Heidolph
Shaker *POLYMAX 2040*	Heidolph
Shaker *VXR Basic Vibrax*	Ika, Germany
Spectrophotometer *NanoDrop ND-1000*	PeqLab, Erlangen, Germany
Thermomixer *comfort*	Eppendorf, Germany
Vacuum pump	IBS Integra Biosciences, Germany
Vortex *Genie 2*	Scientific Industries, Bohemia, NY, USA

II.1.2 Consumables

Table II.1.2 Consumables

Product	**Company**
96-well plates for microscopy, clear bottom	Corning, Corning, NY, US
96-well plates for luminometer, white bottom	Perkin Elmer, US
Cell culture dishes (10 cm, 15 cm)	Greiner, Frickenhausen, Germany
Cell culture plates (6-well, 12-well)	Greiner
Cell scraper (16 cm, 25 cm)	Sarstedt, Germany
Cryo tubes *Cryoline*	Nunc, Thermo Scientific
Pipet tips (10 µL, 20-200 µL, 1,000 µL)	Greiner
Protran nitrocellulose transfer membrane	Whatman, Dassel, Germany
Reaction tube (0.5 mL, 1.5 mL, 2.0 mL)	Eppendorf
Reaction tube (15 mL, 50 mL)	Greiner
Whatman paper	Whatman

II.1.3 Chemicals and reagents

Table II.1.3 Chemicals and reagents

Substance	Company
Albumin Fraction V (Bovine Serum Albumine)	Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Roth
Calcium chloride dihydrate ($CaCl_2$ x $2H_2O$)	Roth
CellTiter-Glo®Reagent	Promega, WI, US
Complete Mini Protease Inhibitor	Roche, Basel, Schweiz
Dimethyl sulfoxide (DMSO)	AppliChem, Darmstadt, Germany
Guava ICF Cleaning Solution	Millipore, Merck
Isopropanol	Th. Geyer, Renningen, Germany
Lipofectamine 2000	Invitrogen, Life Technologies
Magnesium chloride ($MgCl_2$) for PCR	Fermentas, Thermo Scientific
$MgCl_2$ hexahydrate ($MgCl_2$ x $6H_2O$)	Roth
Methanol >99% (MetOH)	Roth
Nuclease free water	Ambion, Life Technologies, CA, US
Ponceau S	Roth
Potassium chloride (KCl)	Roth
Potassium hydrogenphosphate (KH_2PO_4)	Roth
Prestained Protein Ladder	Fermentas, Thermo Scientific
Propidium iodide (PI)	Sigma-Aldrich, MI, US
Rotiphorese Gel 30	Roth
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Roth
Sodium-hydrogenphosphate-heptahydrate (Na_2HPO_4 x $7H_2O$)	Roth

TetraCycline	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Roth
Thymidine	Sigma-Aldrich
Trisamine (Tris)	Roth
Triton X-100	Applichem
Tween 20	Applichem

II.1.4 Buffers and solutions

Table II.1.4 Buffers and solutions

Cell lysis buffer

Urea	2.5 M
RIPA lysis buffer	100%
⇨for SDS PAGE, diluted with 6x Laemmli 1:5	

PBS^{++}

NaCl	24.00 mM
KCl	0.27 mM
Na_2HPO_4 x $7H_2O$	0.81 mM
KH_2PO_4	0.15 mM
$CaCl_2$ x $2H_2O$	1.00 mM
$MgCl_2$ x $6H_2O$	0.50 mM
dissolved in H_2O	

Laemmli buffer, 6x

Tris pH 6.8	0.35 M
Glycerin	30.00%
SDS	10.00%
Dithiotreitol	9.30%
Bromophenol blue	0.02%
dissolved in H_2O	

Phosphat buffered saline (PBS), pH 7.5

NaCl	24.00 mM
KCl	0.27 mM
Na_2HPO_4 x $7H_2O$	0.81 mM
KH_2PO_4	0.15 mM
dissolved in H_2O	

Ponceau S solution

Ponceau S	0.5%
Acetic acid	1.0%
dissolved in H_2O	

RIPA lysis buffer, pH 7.5

Triton X-100	1.0%
Na deoxycholate	1.0%
SDS	0.1%
NaCl	150 mM
EDTA	10 mM
Tris, pH 7.5	20 mM
Trasylol	50,000 KIU
dissolved in H_2O	

SDS running buffer

Tris	25.0 mM
Glycin	86.1 mM
SDS	3.5 mM
dissolved in H_2O	

Tris buffered saline + Tween 20 (TBST), pH 7.6

Tris	50 mM
NaCl	150 mM
Tween 20	0.1%
dissolved in H_2O	

Western blot blocking solution

BSA or milk powder	5%
dissolved in TBST	

Western blot buffer, pH 8.3

Tris	25 mM
Glycin	192 mM
MetOH	20%
dissolved in H_2O	

II.1.5 Chemotherapeutics and pharmacological inhibitors

Table II.1.5 Chemotherapeutics

Name	Systematic name	Company
Gemcitabine	2',2'-difluorodeoxycytidine (dFdC)	Eli Lilly, IN, US

Table II.1.6 Pharmacological inhibitors

Inhibitor	Commercial name	Target	Company
ATRi	VE-821	ATR	Selleckchem
Chk1i	SB-218078	Chk1	Calbiochem, Merck
Nutlin-3	Nutlin-3	Mdm2	Sigma-Aldrich
RO-3306	RO-3306	Cdk1	Sigma-Aldrich
Wee1i	MK-1775	Wee1	Selleckchem

II.1.6 Kits

Table II.1.7 Kits

Name	Company
Guava Check Kit	Millipore, Merck
Immobilon Western HRP Substrate Peroxide Solution	Millipore, Merck
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Scientific
CellTiter-Glo®Luminescence Cell Viability Assay	Promega

II.1.7 Antibodies

Table II.1.8 Primary antibodies

Target	Clone	Source organism	Dilution immunoblotting	Company
Cdc2	POH-1	mouse	1:2,000	CST, Beverly, MA, USA
Cdc2 pY15		rabbit	1:1,000	Abcam
Chk1	2G1D5	mouse	1:1,000	Cell Signaling Technology
Chk1 pS317		rabbit	1:1,000	Cell Signaling Technology
H2AX pS319	JBW301	mouse	1:4,000	Millipore, Merck
H2AX pS319		rabbit	1:1,000	Cell Signaling Technology
H3 pS10	(D2C8) XP	rabbit	1:1,600	Cell Signaling Technology
HSC70	B-6	mouse	1:15,000	Santa Cruz Biotechnology
Mdm2	(Ab-1), IF-2	mouse	1:300	Calbiochem
p21	(Ab-1) EA10	mouse	1:500	Calbiochem
p53	DO-1	mouse	1:1,000	Santa Cruz Biotechnology
PARP		rabbit	1:1,000	Cell Signaling Technology
Rad17pS645	D5H5	rabbit	1:1,000	Cell Signaling Technology
Wee1		rabbit	1:1,000	Cell Signaling Technology
β-Actin	AC-15	mouse	1:20,000	Abcam

Table II.1.9 Secondary antibodies

Antibody	**Cat. Number**	**Company**
HRP-coupled AffiniPure F(ab')2 fragment, anti-mouse IgG (H+L)	711-036-152	Jackson Immunoresearch, Europe, Newmarket, UK
HRP-coupled AffiniPure F(ab')2 fragment, anti-rabbit IgG (H+L)	715-036-150	Jackson, Immunoresearch

II.1.8 Human cell culture

Table II.1.10 Cell lines

Cell line	Origin
HeLa	Cervical adenocarcinoma
PANC-1	Pancreatic epithelioid carcinoma
U2OS	Osteosarcoma

Table II.1.11 Cell culture reagents

Reagent	Company
Ciprofloxacin	Bayer
Dulbecco's Modified Eagle Medium (DMEM), powder	Gibco, Life Technologies
Fetal Calf Serum (FCS)	Gibco, Life Technologies
L-Glutamine	Gibco, Life Technologies
PBS (tablets)	Gibco, Life Technologies
Penicillin/Streptomycin	Gibco, Life Technologies
Tetracycline	Gibco, Life Technologies
Trypsin/EDTA	Gibco, Life Technologies

DMEM

DMEM, powder	10.0 g
$NaHCO_3$	3.7 g
HEPES	5.96 g
dissolved in H_2O	

II.1.9 Software

Table II.1.12 Lab Software

Name	Company
Celigo Software	Cyntellect
Excel	Microsoft, Redmond, WA, United States
Guava Express Software	Millipore, Merck
INTAS lab ID	Intas Science Imaging Instruments
NanoDrop Software	Peqlab
Adobe Photoshop CS5	Adobe Systems, San Jose, CA, United States

The “Materials” part was adapted from the PhD thesis “Combining gemcitabine with checkpoint kinase inhibitors to sensitize pancreatic tumors” by Dr. Priyanka Saini, Göttingen 2014, Dobbelstein group.

https://ediss.uni-goettingen.de/bitstream/handle/11858/00-1735-0000-0022-5FB7-B/final%20thesis%20for%20publication%20no%20cv.pdf?sequence=1

II.2 Methods

II.2.1 Cell culture work

II.2.1.1 Human cell culture

For our *in vitro* experiments, immortalized adherent human cell lines were cultured in cell culture dishes at 37°C and 5% CO_2 under humidified conditions. For cell splitting, cells were shortly washed with 1xPBS and then treated with trypsin. After stopping the reaction with full medium, the cells were sub-cultured at the desired ratio.

Table II.2.1 Cell culture media recipes

Cell lines	Media	Supplements
U2OS (Osteocarcinoma)	DMEM	FCS, L-Glutamine, Penicillin/Streptomycin, Ciprofloxacin, Tetraycline
Panc1 (Pancreatic tumor)	DMEM	FCS, L-Glutamine, Penicillin/Streptomycin, Ciprofloxacin, Tetraycline
HeLa (Cervical cancer)	DMEM	FCS, L-Glutamine, Penicillin/Streptomycin, Ciprofloxacin, Tetraycline

II.2.1.2 Long term storage of cells

For long term storage of cells, confluent cell culture plates with low passage numbers were trypsinized and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was removed and the cells were then resuspended in previously ice cooled freezing medium, which consists of FCS/DMSO in a 9:1 ratio. The cells were aliquoted into cryo-vials, frozen at -80°C, and afterwards transferred into liquid nitrogen for long term storage.

II.2.1.3 siRNA reverse transfection of cells

For an efficient siRNA transfection of cells, the reverse-transfection approach was used: Adherent cells were trypsinized and the cell density was adjusted to 80.000 cells / ml. siRNAs and Lipofectamine 2000 (LF 2000) were diluted in DMEM without supplements as mentioned in Table II.2.2.

Table II.2.2 siRNA transfection protocol

Plate Format	Cell number* (U2OS)	Medium (µl)	siRNA (50 µM)	Medium (µl)	LF2000 (µl)
6 well	160,000	200	0,6 µl (10 nM)	200	4

The prepared dilutions were incubated for 5 min at room temperature and then mixed in a 1:1 ratio and further incubated for 20 min. After incubation, the mixture was combined with 2ml of cell suspension into a well of a cell culture dish. The medium was exchanged after 24 h and the cells were either treated or harvested for further experiments.

Table II.2.3 Small interfering RNAs

Name (Silencer select, Ambion)	siRNA ID
Negative Control	Undisclosed
Wee1	s21
Mus81	s37038
Claspin	s34330
Cdk1-1	s464
Cdk1-2	s465

II.2.1.4 Chemical or drug treatment

Table II.2.4 Compound concentrations

Inhibitor	Target	Solvent	Stock concentration	Working concentration
SB 218078	Chk1	DMSO	2.5 mM	2.5 µM/ 5 µM
VE-821	ATR	DMSO	10 mM	10 µM/ 5 µM
MK-1775	Wee1	DMSO	1 mM	1 µM/ 0.5 µM
RO-3306	CDK1	DMSO	10 mM	10 µM
Nutlin-3	Mdm2	DMSO	20mM	8 µM

Chemotherapeutic Drug	Solvent	Stock concentration	Working concentrations
Gemcitabine	Water	64 mM	300/ 25/ 5 nM

II.2.2 Protein Biochemistry

II.2.2.1 Preparation of whole cell lysates

The entire protein extraction protocol was conducted on ice. Cells were mechanically brought into suspension by scraping and were transferred into a 2 ml tube, which was then centrifuged at 4400 rpm for 4 min at 4 °C. After removal of the supernatant, the cells were washed with 1 ml 1xPBS and centrifuged again. Finally, the cells were again resuspended in 90 µl of freshly prepared lysis buffer and strongly shaken for 30 min at 4°C. Before use, the samples were centrifuged at 13,200 rpm for 13 min to pellet the DNA.

The protein concentration of the samples was measured using the bicinchoninic acid assay (BCA assay) kit. This colorimetric assay measures the color reaction of the substrate with the protein. The kit reagents A and B were mixed in a 49:1 ratio. 5 µl of the protein sample to be measured is added to 95 µl of the substrate mixture and incubated at 37 °C for 30 min. The samples were then measured using a spectrophotometer and referenced to a standard curve for an estimated protein concentration.

II.2.2.2 Separation of proteins by SDS-PAGE

For gel electrophoresis, a loading dye (6 x Laemmli buffer) was added to the sample for a 1 x final concentration. The samples were then boiled 10 min at 95°C for protein linearization. The acrylamide gel consists of a high percentage and a low percentage part: The low percentage component concentrates the protein sample from the loading pocket, whereas the high percentage part of the gel separates the proteins according to size. The components of the two gels are summarized in Table II.1.8. The prepared protein samples were loaded into the well pockets alongside a protein marker for size determination and run at a voltage of initially 100 V and later 130 V, until the desired separation has been achieved.

Table II.2.5 Acrylamide gel protocol

	Stacking gel	Resolving gel
Acrylamide/bisacrylamide	5%	6-12%
1M Tris, pH 6.8	126 mM	-
1.5M Tris, pH 8.8	-	375 mM
10% SDS	0.1%	0.1%
10% APS	0.1%	0.1%
TEMED	0.3%	0.4%

II.2.3 Western blotting

The separated proteins in the gel were blotted onto a nitrocellulose membrane, the transfer was conducted at 100 V for 120 min in the cold room. The membrane was then Ponceau S stained, scanned for archiving, and subsequently blocked with blocking buffer for 30 min. The membranes were incubated overnight in primary antibodies (refer to Table II.1.8), on a rotator at 4°C. The next day, a secondary antibody, which specifically targets the primary antibody, is applied to the membrane in a 1:10,000 dilution for 1h on a rotator at room temperature. These secondary antibodies are further coupled to a horse-radish peroxide enzyme, which is able to turn over the substrate to produce a luminescent product. The protein amount can thus be estimated through a light signal.

II.2.3 Cell biology methods

II.2.3.1 Cell proliferation assay

To visualize proliferation rates, adherent cells were seeded in 96 well plates and monitored over time with an automated optical microscope, the *Celigo cell cytometer.* This imaging of living cells allows access to their different growth rates in different chemical environments.

The cells were treated with chemical inhibitors and chemotherapeutic drugs for 24h and the medium was exchanged 24h after treatment. The culture medium in gemcitabine treated samples was exchanged twice. The plates were measured daily at approximately the same time points to record the confluency status of each well. The medium was exchanged every second day. For analysis, confluency was plotted against time. The experiment was conducted as technical triplicates.

II.2.3.2 Cell Viability Assay

Quantification of ATP can be used as a marker for cell viability in cultured cells. Using the *CellTiter-Glo®Luminescence Cell Viability Assay* (Promega), the number of metabolically active cells can be assessed through cellular lysis and subsequent conversion of ATP into a light signal.

In preparation for the assay, cells were seeded into white 96 well plates and treated with different chemicals after 24h. After another 72h, cells were lysed by addition of the CellTiter-Glo®Reagent in a 1:1 ratio. The plate was gently shaken in the dark for 10 min and subsequently measured using the *Luminometer DLReady™Centro LB 960* plate reader. The experiments were conducted as technical triplicates.

II.2.3.3 Cell cycle analysis

Using flow cytometry, the DNA content of a single cell can be evaluated. By pooling the DNA contents of all cells in a well, one can access the cell cycle profile of the entire cellular population. After permeabilization of cells, the DNA content can be visualized by the fluorescent dye Propidum iodide, a DNA intercalating substance. The amount of the PI signal in the cell indicates its cell cycle status, 1n for G1 and 2n for G2, S phase cells between 1n and 2n.

Cells in 6 well plates were pretreated with Nutlin-3a for 24h. Subsequently, the cells were treated with Wee1 inhibitor and gemcitabine in addition to Nutlin-3a. After another 24h, the samples were harvested with trypsin and centrifuged at 1000rpm for 5 min at 4°C. The supernatant was removed and the cells were resuspended in ice-cold 1xPBS. Ice-cold ethanol was added slowly in drops, while vortexing the sample, to the final volume of 2ml. The cells were fixed at -20°C for the minimum of 1 hour or overnight and longer. For analysis preparation, the samples were spun at 2,4k rpm for 5 min at 4°C and subsequently rehydrated in 1ml 1xPBS++ on ice for 10 min. The cells were again centrifuged and RNAse digested in 100 µl 0.5 mg/ml RNAse A in 1xPBS++ at 37°C for 30 min. The RNAse working solution was previously DNAse inactivated at 70°C for 10 min. The samples were diluted and sieved before addition of 3 µl 30 µg/ml PI to 100 µl of cell suspension and finally loaded for flow cytometry using the *Guava PCA-96 Base System*.

The “Methods” part was in part adapted from the PhD thesis “Combining gemcitabine with checkpoint kinase inhibitors to sensitize pancreatic tumors” by Dr. Priyanka Saini, Göttingen 2014, Dobbelstein group.

https://ediss.uni-goettingen.de/bitstream/handle/11858/00-1735-0000-0022-5FB7-B/final%20thesis%20for%20publication%20no%20cv.pdf?sequence=1

III Results

III.1 The mechanism of Wee1i – gemcitabin mediated synergistic lethality in cancer cell lines

III.1.1 The combination treatment of Wee1i and gemcitabine is highly efficient in killing transformed cells

To evaluate their combinatory effects on cancer cell lines, U2OS (osteosarcoma) and Panc1 (pancreatic adenocarcinoma) cells were treated with pharmacological small molecule inhibitors against Chk1, Wee1 and ATR in combination with gemcitabine. In the assay conducted, we have measured the increase in cell confluency over 8-13 days after treatment via an automated optical microscope (Celigo Cytometer). Cells were treated with different inhibitors in addition with gemcitabine, the medium was exchanged after 24h and confluency measurements were taken daily. We have observed that combining inhibitors of either Wee1 or ATR with gemcitabine leads to a slower growth of the cells compared to the Chk1 inhibitor gemcitabine combination in both Panc1 and U2OS cells (see Figure III.1). This effect seems to be p53 independent, as U2OS cells are p53 wildtype and Panc1 cells are p53 DNA binding mutants.

III.1.2 The Wee1 inhibitor – gemcitabine combination reduces Chk1 phosphorylation over time

After observing a synergistic effect on cell proliferation in a Wee1 inhibitor – gemcitabine co-treatment situation, we wanted to understand whether inhibition of the Wee1 kinase affects DNA damage signaling directly. We have therefore conducted a Chk1 activation time course experiment and evaluated early effects of the inhibitor treatment by immunoblot analysis. As a result, the combination of Wee1 inhibitor and gemcitabine clearly upregulates phosphorylation of yH2AX when compared with the gemcitabine single treatment condition at all observed time points (see Figure III.2). Furthermore, a 12h inhibition of Wee1 is sufficient to reduce phosphorylation of Chk1 at Ser317, which becomes clearly visible at the 24h. Due to the delayed decrease of Chk1 phosphorylation, we suggest not a direct but an indirect function of the Wee1 kinase to maintain Chk1 phosphorylation. We have also observed cleaved PARP in the 24h treated sample, suggesting apoptosis to occur in the Wee1 inhibitor – gemcitabine co-treated cells. For validation of the functionality of our Wee1 inhibitor we detected a decrease at the previously described Wee1 phosphorylation site on Cdk1 at Tyr15 (Parker and Piwnica-Worms 1992) when treated with the inhibitor (see Figure S1). Furthermore we have observed the same effects on the DDR pathway when transfecting a siRNA targeting Wee1 and subsequently treating with gemcitabine (see Figure S2). In conclusion, we can observe a strong synergistic effect on yH2AX at all time points investigated and a decreased phosphorylation of Chk1 in Wee1 inhibitor – gemcitabine co-treated cells after 24h.

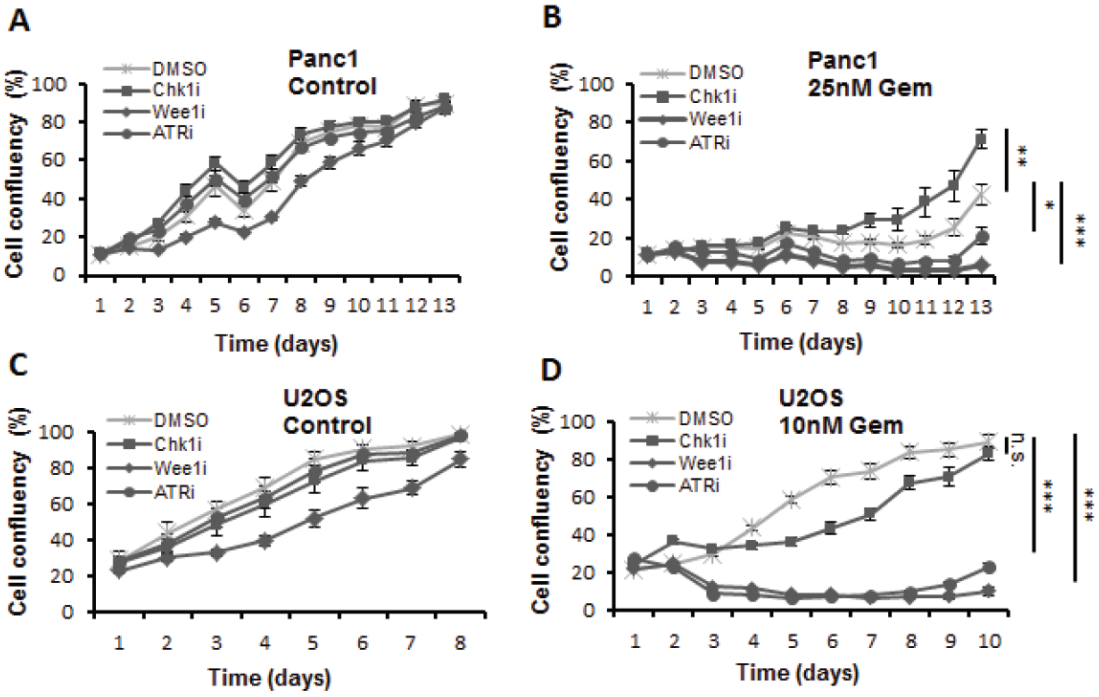


Figure III.1: Inhibitors of Chk1, Wee1 and ATR kinases enhance gemcitabine mediated cytotoxicity. The cells were treated for 24h with Chk1 (2.5µM), Wee1 (0.5µM) and ATR (5µM) inhibitors with or without gemcitabine (Gem, 10nM). The confluency of the wells was monitored daily for one or two weeks. The error bars represent standard deviation, n=3. p-values (based on Student's t-test, 2-sided, assuming different variances) were determined for the last measurement of the respective experiment. The experiments were performed by Dr. Priyanka Saini.

III.1.3 Wee1 inhibition effects on DDR are not due to induction of apoptosis

As we have observed PARP cleavage in the Wee1 inhibitor – gemcitabine co-treatment (see Figure III.2), induction of apoptosis could lead to yH2AX phosphorylation as previously described (Rogakou et al. 2000). Apoptosis also induces the Chk1 phosphatase PP2A (Santoro et al. 1998; Leung-Pineda et al. 2006), we therefore had to rule out the contribution of apoptosis to our Wee1 kinase inhibition – Chk1 dephosphorylation effect. U2OS and Panc1 cells were subjected to the Wee1 inhibitor – gemcitabine combination treatment in the presence of the caspase inhibitor Z-VAD, which potently inhibits the apoptotic pathways (Garcia-Calvo et al. 1998). Our western blot results suggest, that Chk1 dephosphorylation upon Wee1 inhibition occurs independently from apoptotic activities in the cell (see Figure III.3).

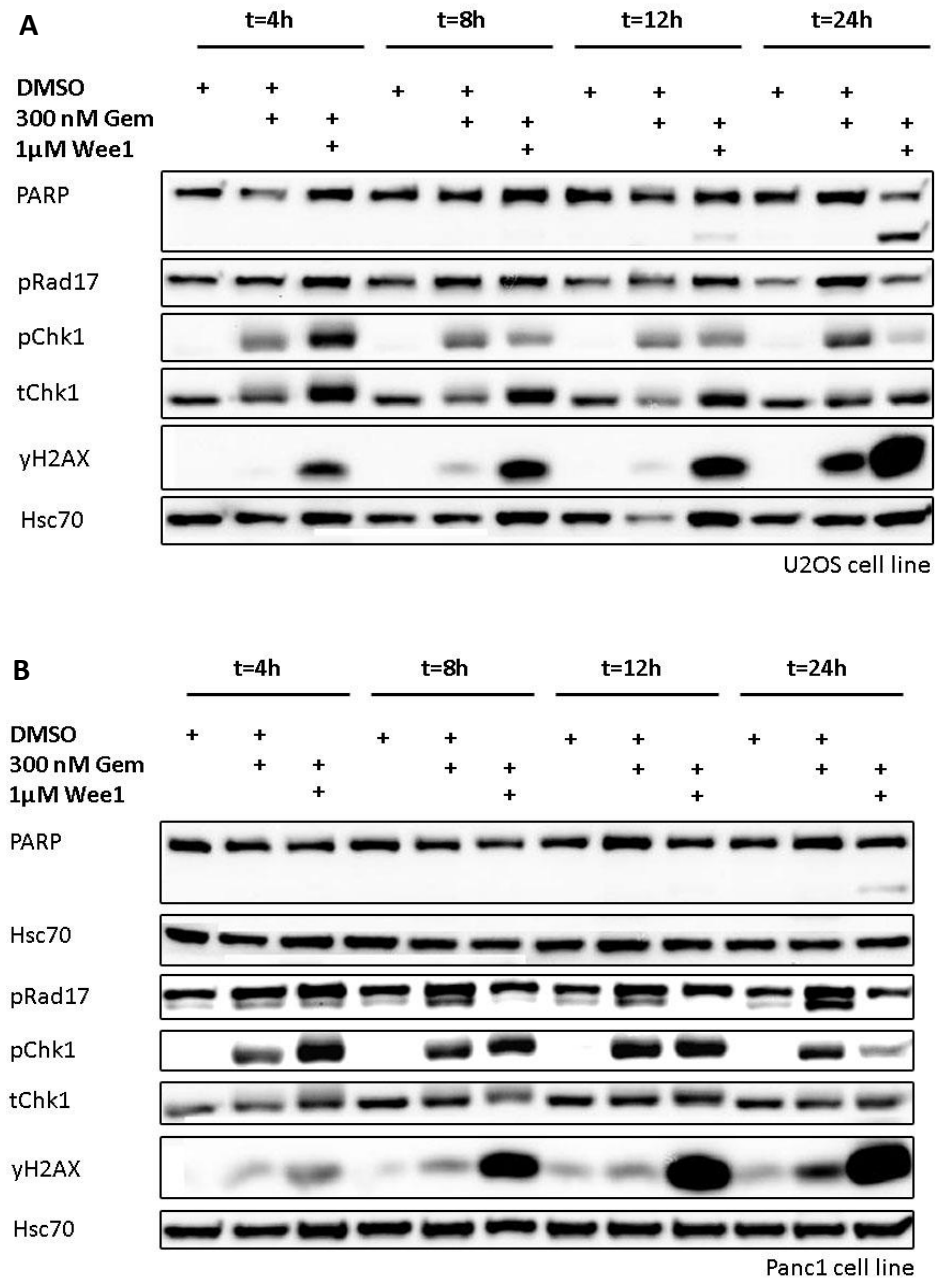


Figure III.2: Wee1 inhibition decreases Chk1 activation in Wee1 inhibitor – gemcitabine co-treated cells in a time dependent manner. U2OS and Panc1 cells were treated with Wee1 inhibitor (MK-1775) and gemcitabine. Samples were harvested according to the time course experiment and prepared for western blotting.

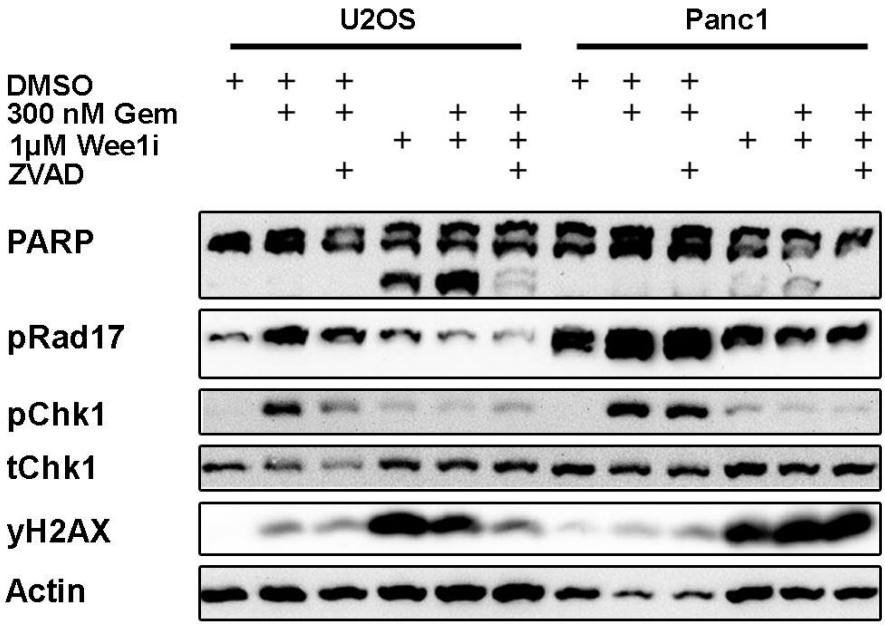


Figure III.3: Decrease in Chk1 phosphorylation is independent of caspase activity. U2OS and Panc1 cells were treated with with Wee1 inhibitor (MK-1775) and gemcitabine, with and without 20 µM of caspase inhibitor Z-VAD. The samples were harvested after 24 h and prepared for western blotting. tChk1, tCdk1 describe total proteins while pChk1, pCdk1 denominate phosphorylated forms.

III.1.4 Cdk1 loss of function rescues Wee1 inhibition-induced decrease of Chk1 phosphorylation

The Wee1 kinase is an important cell cycle gatekeeper for the G2/M phase transition, as it is able to phosphorylate Cdk1 at the inhibitory Tyr15 phosphorylation site (Parker et al. 1992). Inhibition of the kinase therefore leads to Cdk1 activation and forces entry into mitosis (Aarts et al. 2012). To investigate whether the decrease of Chk1 inhibition upon Wee1 inhibition is due to Cdk1, we have used the ATP-competitive small molecule inhibitor RO-3306 against Cdk1 (Vassilev et al. 2004) in combination with the Wee1 inhibitor MK-1775 and gemcitabine. Upon this treatment, the phosphorylation of Chk1 was restored (see Figure III.4). In line with these observations, the removal of Cdk1 by siRNAs also restored Chk1 phosphorylation upon simultaneous knockdown of Wee1 in the presence of gemcitabine (see Figure III.5). In conclusion, Cdk1 is specifically required for inactivating the ATR-Chk1 pathway upon Wee1 inhibition.

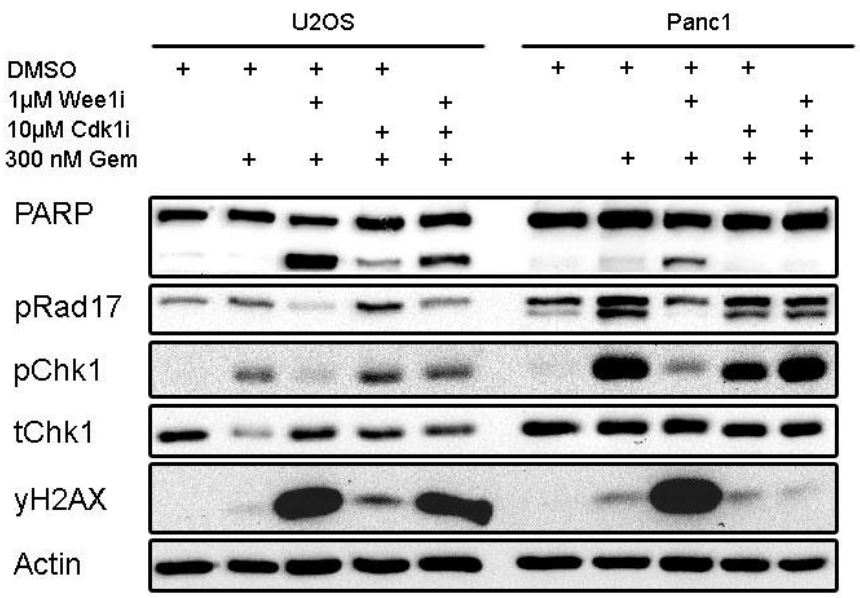


Figure III.4: Decrease in Chk1 phosphorylation in Wee1i treated cells is mediated by Cdk1. Cells were treated with Cdk1 and Wee1 inhibitors in the presence of gemcitabine. The samples were harvested after 24 h and prepared for western blotting.

III.1.5: Wee1 inhibition induced decrease of Chk1 phosphorylation through Cdk1 is independent from pRb

The inactivation of the Retinoblastoma protein (pRb) has been shown to be regulated by various Cyclin-Cdk complexes (Lundberg and Weinberg 1998). To investigate whether pRb contributes to the effects of Wee1 inhibition on DNA damage signaling, we have tested the Cdk1 and Wee1 inhibitor with gemcitabine in the HeLa cell line, in which pRb has been inactivated by the E7 viral protein (Gonzalez et al. 2001). As a result, even in HeLa cells Cdk1 inhibition is able to rescue Chk1 phosphorylation upon Wee1 kinase inhibition (see Figure III.6). We therefore suggest, that Wee1 inhibition effects on Chk1 phosphorylation are dependent on Cdk1, but independent of pRb.

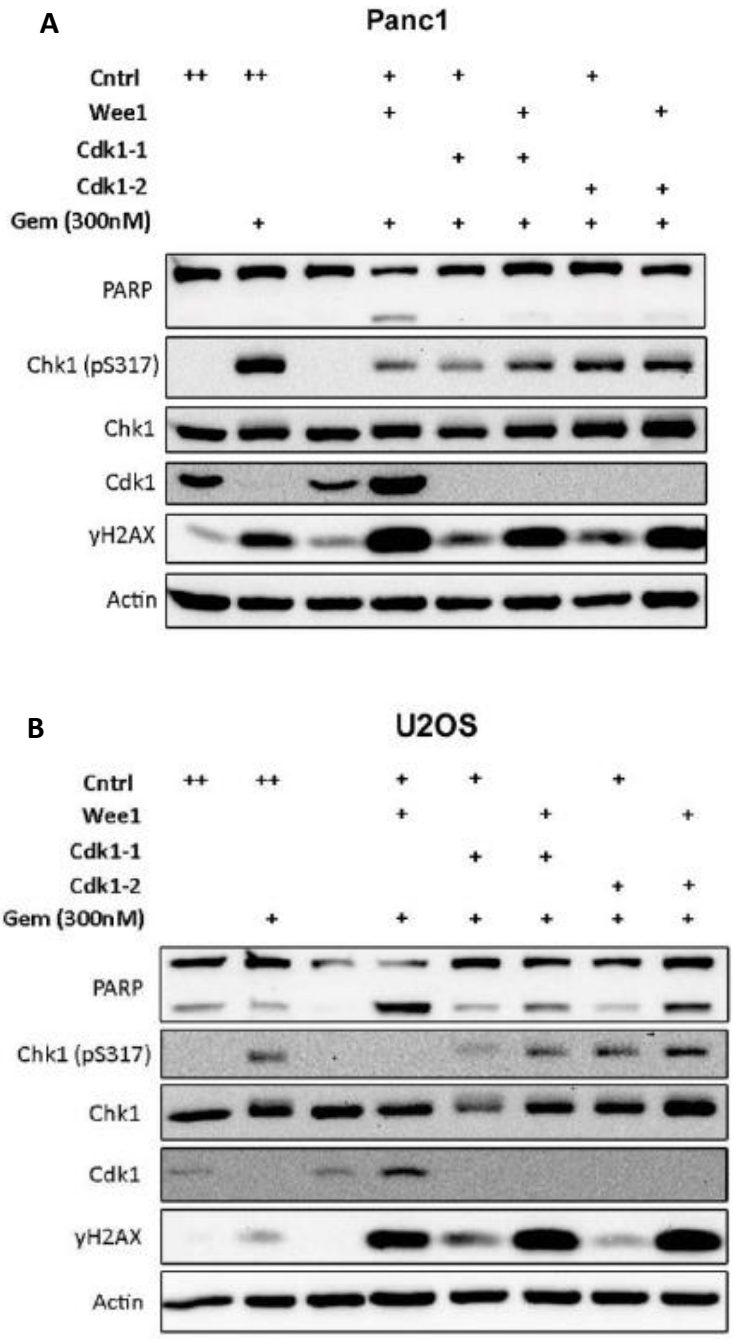


Figure III.5: siRNA mediated knockdown of Cdk1 restores the phosphorylation of Chk1 upon simultaneous knockdown of Wee1 and treatment with gemcitabine. 10nM of each siRNA (Cdk1, Wee1, scrambled) was used for transfecting cells. After 48 h of transfection, 300 nM gemcitabine was added for another 24 h. The cells were then harvested and immunoblotted.

III.2 How can we possibly protect untransformed cells from the highly potent Wee1i – gemcitabine chemotherapeutic combination?

III.2.1 Mdm2 inhibition benefits cell survival upon treatment with Wee1 inhibitor and/or gemcitabine

To assess whether pretreatment with an Mdm2 inhibitor affects the survival of p53-proficient cells upon chemotherapeutic treatment, we first incubated U2OS cells with Nutlin-3a for 24h. The cells were then treated again with Nutlin-3a and in addition with gemcitabine and/or Wee1 inhibitor for another 24h. Subsequently, all drugs were removed, and the cell density was monitored by automated optical microscopy (Celigo) for 12 days. Gemcitabine and Wee1i single treatments only moderately prevented cell growth (see Figure III.7A). However, when applied in combination, the two drugs strongly restricted cells proliferating towards confluency. Nutlin-3a pretreatment was able to increase confluency percentages in all drug combinations, therefore protecting p53-proficient cells from the cytotoxic effects of the chemotherapeutic compounds.

Next, we investigated whether Nutlin-3a pre-treatment also affects cell viability when cells are subsequently exposed to gemcitabine and/or Wee1i. Therefore U2OS cells were pre-treated with Nutlin-3a for 24 h, followed by 72 h incubation with Nutlin-3a, gemcitabine and/or Wee1i. The cells were then lysed and a viability assay based on the determination of cellular ATP levels by luciferase was performed (see Figure III.7B). As a result, the viability was reduced in all three single drug treatments, most strongly in the Wee1 inhibitor treated condition. Importantly, Nutlin-3a was able to rescue the viability of Wee1i-treated cells, with or without gemcitabine. Thus, Nutlin-3a pretreatment strongly protects cells from Wee1 inhibitor induced cytotoxicity.

III.2.2 Mdm2 inhibition reduces the DNA damage response and decreases caspase activity upon Wee1 inhibition

To further characterize our finding that Nutlin-3a is able to protect against the gemcitabine-Wee1 inhibitor combination induced cytotoxicity, we have analyzed cell lysates via western blotting. U2OS cells were incubated with Nutlin-3a for 24h. Subsequently they were then treated again with Nutlin-3a, in addition with gemcitabine and/or Wee1 inhibitor for another 24h. The cells were then harvested and the lysates were subjected to immunoblotting. Western blot analysis showed that γH2AX, cleaved PARP and phospho-H3 levels decrease upon Nutlin-3a pretreatment, indicating a reduced DNA damage response, apoptosis and mitotic activity within the Nutlin-3a treated cell population in comparison with the untreated sample (see Figure III.8A). Furthermore, as expected, p53 was stabilized upon Nutlin-3a pre-treatment and the p53 downstream effector protein p21 was induced (see Figure III.8B), indicating that the effects are indeed p53-dependent.

III.2.3 Nutlin-3a protection against Wee1 inhibition is dependent on the p53 status of the cell

To show that the Nutlin-3a protection against the gemcitabine – Wee1 inhibitor combination therapy is p53 dependent, we have conducted similar experiments in a p53 proficient/deficient isogenic pair of the HCT116 human colon carcinoma cell line. We treated HCT116 cells with wild-type p53 (HCT116wtp53) and HCT116 lacking p53 (HCT116p53−/−) at the same conditions as previously described for western blotting in U2OS cells. As a result, Nutlin-3a pretreatment protected HCT116wtp53 cells against cytotoxic effects of the gemcitabine – Wee1 inhibitor combination therapy (see Figure III.9). In contrast, HCT116p53−/− were not protected in a Nutlin-3a-dependent manner. These results provide further proof that the observed effects are strictly p53 dependent.

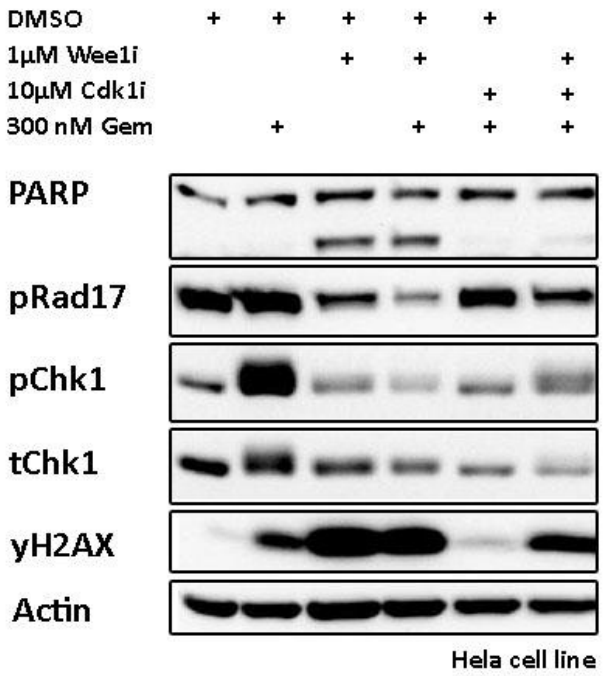
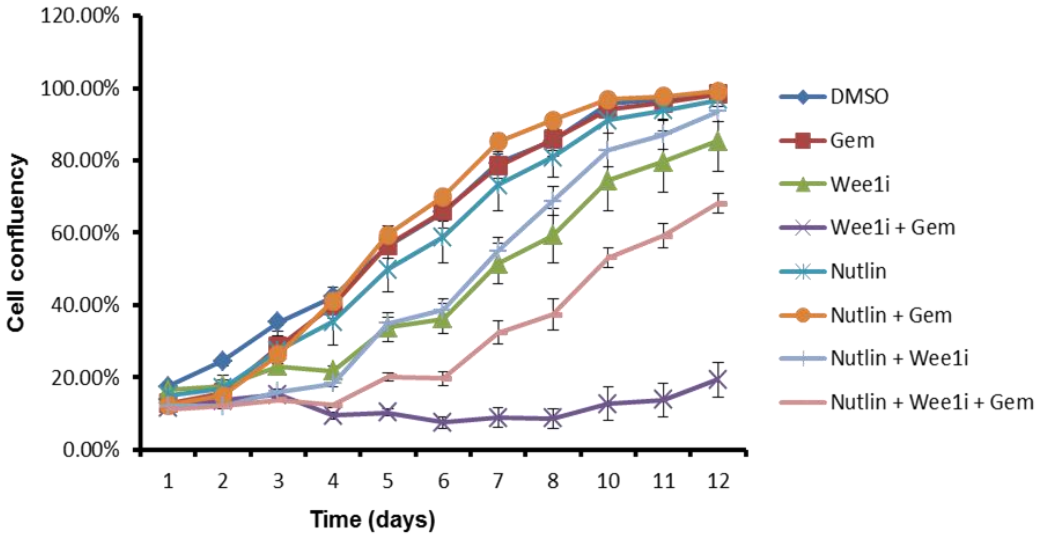


Figure III.6: Wee1 inhibition decreases Chk1 phosphorylation independent from pRb. HeLa cells were treated with Wee1 and Cdk1 inhibitors with gemcitabine for 24h. The samples were then harvested and prepared for western blot analysis.

A



B

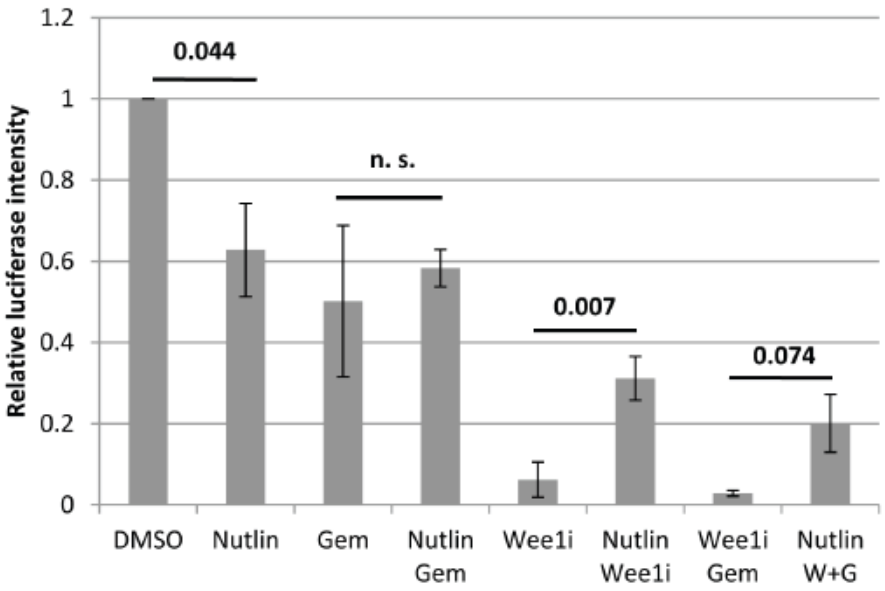


Figure III.7: Nutlin-3a protects cells against Wee1 inhibition and/or gemcitabine. (A) U2OS were treated with Nutlin-3a for 24 h, followed by treatment with 4µ M Nutlin-3a, 300 nM gemcitabine and/or 1µM Wee1 inhibitor for 24 h. The confluency of each well was monitored for 12 days. Error bars represent the standard deviation (n=3). (B) Cells were treated with Nutlin-3a (8 µM) for 24 h, and subsequently treated with Wee1 inhibitor and gemcitabine, along with continuous treatment with 8 µM Nutlin3-a. At 72 h cells were lysed using the CellTiter-Glo®Reagent, and cell viability was measured via an ATP-dependent luciferase signal. Student's t-test p-values are stated above the horizontal bars. Error bars represent the standard error, n=3.

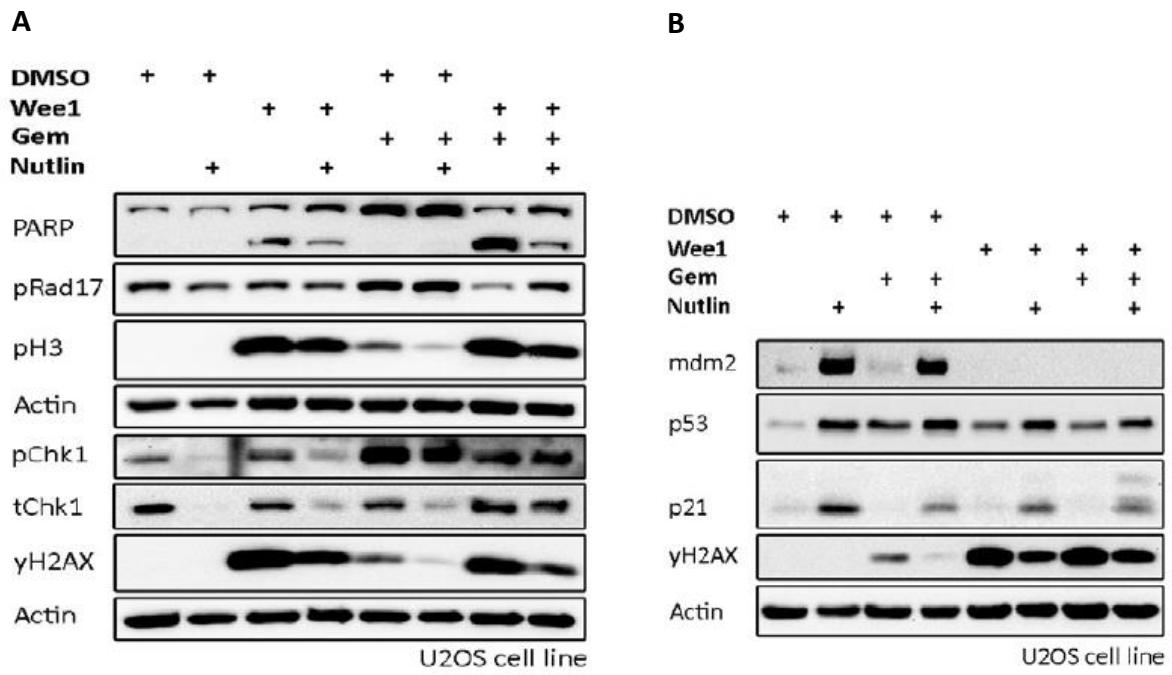


Figure III.8: Nutlin-3a protects cells against the gemcitabine – Wee1 inhibitor co-treatment. Cells were treated with Nutlin-3a (8μM) for 24h, followed by treatment with Wee1 inhibitor and gemcitabine with Nutlin-3a for another 24h. The samples were then harvested and prepared for western blot analysis.

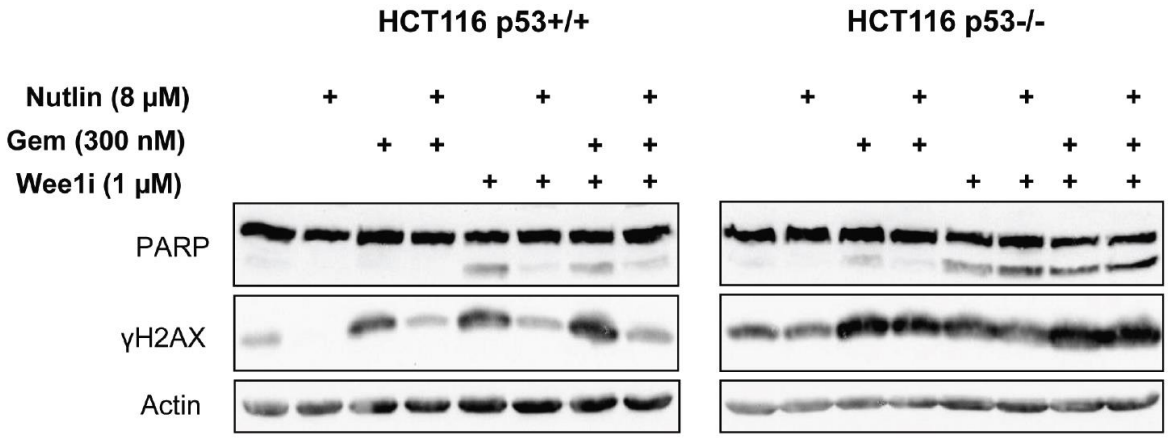


Figure III.9: Nutlin-3a mediated protection against Wee1i/gemcitabine co-treatment is p53 dependent. HCT116 cell lines with different p53 status were pretreated with Nutlin-3a for 24 h, followed by treatment with Wee1 inhibitor and gemcitabine with Nutlin-3a for another 24h. The samples were then harvested and prepared for western blot analysis. Experiments performed by Priyanka Saini.

III.2.4 Nutlin-3a treatment triggers activation of cell cycle checkpoints

It has been previously shown that treatment with Nutlin-3a halts the cell cycle in a p53-dependent manner, reducing the number of cells in S-phase (Miyachi et al. 2009). To investigate whether this is also true for our experimental system, especially in combination with the Wee1 inhibitor MK-1775, we have performed a cell cycle analysis using flow cytometry technology. As a result, Nutlin-3a was indeed able to reduce the number of cells in S-phase, enriching cellular populations at G1 and G2 phases with a 1n and 2n DNA content respectively (see Figure III.10). Most interestingly this was also the case for the combination treatments with gemcitabine and/or Wee1 inhibitor. We therefore suggest that the protective function of Nutlin-3a treatment arises from the cellular exclusion from the chemo-sensitive S-phase.

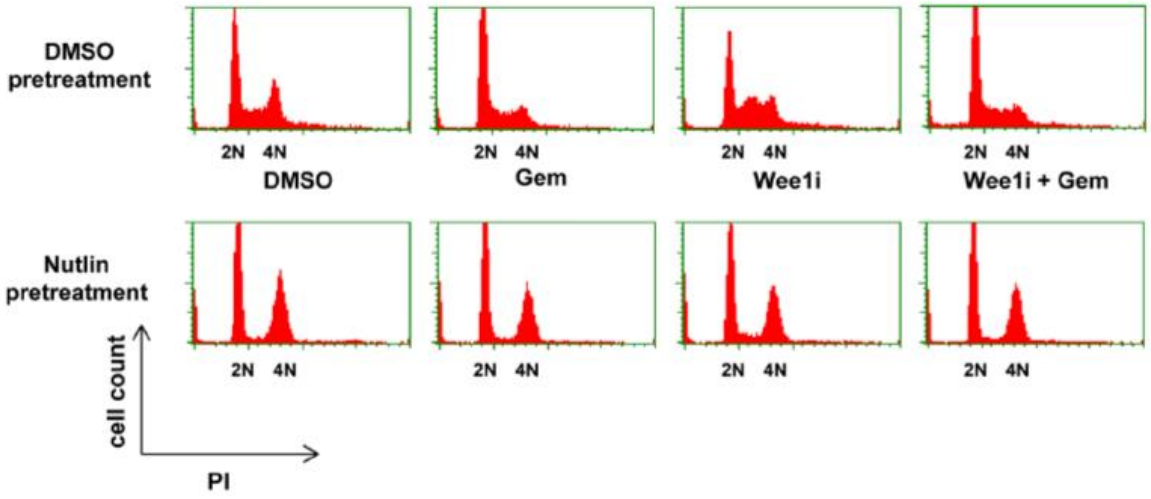


Figure III.10: Nutlin-3a reduces the amount of cells in S phase. Cells were pretreated with Nutlin-3a for 24 h, followed by treatment with Wee1 inhibitor and gemcitabine with Nutlin-3a for another 24 h. Subsequently, the DNA content was assessed by flow cytometry analysis. Histograms depict the relative number of cells found within a small window of DNA content, as determined by propidium iodide stain.

IV Discussion

IV.1 Chemotherapy: Where will we go?

The cancer diseases remain a frontier of our modern time. Their incidence will increase in industrialized countries with their aging populations, as cancer is mainly a disease of the elderly (Rahib et al. 2014), matching up with the cancerogenesis hypothesis of accumulated mutations (Vogelstein et al. 2000). Therefore it is the responsibility of these countries and their politicians, scientists and industrials to improve the cancer treatment situation, to meet the demands of this upcoming and growing cohort of patients.

So how can we go about this problem? Until today, three main categories of treatment have established themselves in western medicine which are steel, ray, and pill. Whereas steel and ray, representing surgical removal and irradiation of tumors, depend on a precise localization of the cancerous tissue, the pill, alias chemotherapy, is a systemic approach to treat a disease which is likely to turn systemic through metastasis of the primary tumor. These three schools of cancer doctors, cancer surgeons, radiotherapists and clinical oncologists, have formed the necessary firm alliances, bringing about a combination of steel, ray and pill to treat a cancer patient. Still, in my opinion, as the steel and the ray are only able to treat the visualized cancer, an even greater importance will be upon chemotherapy, the only currently possible treatment for late stage metastasized tumors, as it represents a systemic approach for a systemic disease.

Chemotherapeutical agents in the early days were chosen merely on empirical evidence (Mukherjee 2011). It was not until major advances in molecular biology that we have begun to understand the mechanisms of their action. Most interestingly, many of these agents are genotoxic towards DNA. It is therefore promising to understand the cellular responses upon DNA damaging agents in detail. DNA damage and repair, including replicative stress and checkpoint regulation, provide a promising research field, to further enhance the efficacy and specificity of established chemotherapeutic agents through combination with novel and specific small-molecule inhibitor compounds. Another approach would be to chemically protect untransformed cells from being targeted by the chemotherapeutic agent, further enhancing the specificity of the treatment.

As we are looking into a dark future with more and more cancer patients on the wards, there might be promise from technological advances of our time: With the establishment of personalized medicine through next-next generation sequencing, each individual patient may soon get his or her individual chemotherapy, which best fits the genotype of the patient and the patients cancer at the time treated. Being cancer researchers of chemotherapy, it is our task to characterize these individual combinatory treatments, so that they are ready when eventually needed.

IV.2 A novel connection between Wee1 and the ATR-Chk1 pathway

As pancreatic tumors tend to grow resistant against gemcitabine (Giovannetti et al. 2006; Nakano et al. 2007), combinatory treatments to further chemo-sensitize these resistant cancers are currently of great interest to improve medical care for this group of patients. Combining cell cycle checkpoint inhibitors with gemcitabine has been shown to further enhance its toxicity (Zabludoff et al. 2008; Prevo et al. 2012). In our hands and in agreement with previous studies (Hirai et al. 2009; Rajeshkumar et al. 2011), the combination of Wee1 inhibition and gemcitabine was potently inhibiting the growth of pancreatic cancer cells (see Figure III.1). What are the mechanisms behind this observation? For one, inhibition of Wee1 ablates the Cdk1 mediated G2/M cell cycle arrest and strongly forces entry into mitosis (Aarts et al. 2012). As gemcitabine delivers a massive amount of replicative stress and thus arrests cells within the S-phase through the intra-S-checkpoint by inhibiting Cdk1 and Cdk2 (Bartek and Lukas 2003; Dobbelstein and Sorensen 2015), it seems logical that cells within the S-phase will be susceptible towards the Wee1 inhibition forced premature mitotic entry, which prevents the inhibitory phosphorylations Tyr15 and Thr14 on Cdk1 by the Wee1 kinase, thus activating Cdk1 and overriding the intra-S-checkpoint (Smith et al. 2010). Indeed we have shown this in a two-dimensional flow cytometry, quantifying both the DNA content and the amount of phosphorylated H3 in each cell, showing that the co-treatment with gemcitabine and Wee1 inhibitor leads to a larger cellular population within the premature mitosis window as compared with the Wee1 inhibitor treatment alone (Li et al. 2015, the experiment was conducted by Dr. Priyanka Saini). Furthermore, within this work, we have observed a significant increase in DNA damage via yH2AX and a reduced activation of the DDR via reduced phosphorylation of Chk1 upon the combination with gemcitabine and the Wee1 inhibitor (see Figure III.2). It has been previously shown that knockdown of the Wee1

kinase leads to stalling of DNA replication forks and generates DNA damage due to activation of the hetero-dimeric Mus81-Eme1 structure-specific endonuclease, which is capable of generating DSBs (Dominguez-Kelly et al. 2011). However, siRNA mediated knockdown of Mus81 neither rescued Wee1 siRNA knockdown induced reduction of Chk1 phosphorylation, nor did it reduce the DNA damage measured via yH2AX (see Figure S3). Therefore, we had to characterize a novel mechanism by which Wee1 loss of function would lead to a decreased phosphorylation of Chk1 while at the same time synergistically accumulating DNA damage when treated with gemcitabine.

IV.2.1 Cdk1 decreases ATR-Chk1 activation upon Wee1 inhibition

Our results show that the Wee1-inhibition-induced reduction in Chk1 phosphorylation is mediated through the activity of Cdk1 (see Figures III.4 and III.5). Therefore, we suggest Cdk1 to play an important role in shutting down the checkpoint activation by ATR. This observation might be of importance for tumor therapies, as Cdk inhibitors are currently being tested in clinical trials (Cicenas and Valius 2011). The combination of Wee1 inhibition and gemcitabine might therefore be an effective approach to treat tumors expressing high levels of Cdks. As there is no reported evidence of direct interactions between Cdk1 and the ATR-Chk1 pathway, we have looked at different Cdk1 downstream factors. Mus81 is such a Cdk1 substrate, and as mentioned above, it does not seem to play a role in rescuing Chk1 phosphorylation upon Wee1 inhibition (see Figure S3). In addition, we have investigated whether pRb impacts the ATR-Chk1 pathway either through its target E2F or through direct protein-protein interaction. Upon Wee1 inhibition, the transcription factor E2F might suppress ATR on mRNA level (Ren et al. 2002). However, we did not observe a difference in transcription of the ATR gene upon Wee1 inhibition when compared with the DMSO treated control in a qRT-PCR experiment (Saini et al. 2015b). Furthermore, pRb does not modulate this pathway on a protein level, as even in HeLa cells which possess an impaired pRb pathway, inhibition of Cdk1 is able to rescue the effects of Wee1 inhibition (see Figure III.6). As a conclusion, effects of the Wee1 inhibitor MK-1775 and gemcitabine combination are independent of Mus81 and the pRb status.

IV.2.2 Unraveling the mechanism

With the data presented I have contributed to the publication from Dr. Priyanka Saini, who has conducted further experiments to unravel the mechanism of the Wee1i – gemcitabine mediated lethality in cancer cells (Saini et al. 2015b). Dr. Saini has shown that Wee1-inhibition-induced and hyper-activated Cdk1 transduces its signal through Polo-like kinase 1 (Plk1) (Yamaguchi et al. 2005), which then activates its effector protein Claspin (Peschiaroli et al. 2006). Claspin binding to Chk1 increases its affinity as an ATR substrate (Chini and Chen 2003). Plk1 mediated Claspin phosphorylation marks it for ubiquitin mediated proteasomal degradation (Peschiaroli et al. 2006), thereby reducing Chk1 phosphorylation through ATR and allowing recovery from an activated DNA replication checkpoint. Furthermore Dr. Saini has found CtIP to be downregulated upon Wee1 inhibition in a Cdk1 dependent manner. CtIP is a factor associated with DNA resection and it also contributes to ATR-Chk1 signaling (Kousholt et al. 2012), thus upholding Chk1 phosphorylation. Taken together, Dr. Saini has unraveled the mechanism of the Wee1i – gemcitabine mediated synthetic lethality in cancer cell lines: The reduction of the DDR pathway, via dephosphorylation of Chk1 through downregulation of the proteins Claspin and CtIP in a Cdk1-dependent manner, leads to an increased susceptibility towards the DNA damage issued by the nucleoside analogue gemcitabine (Saini et al. 2015b, see Figure IV.1).

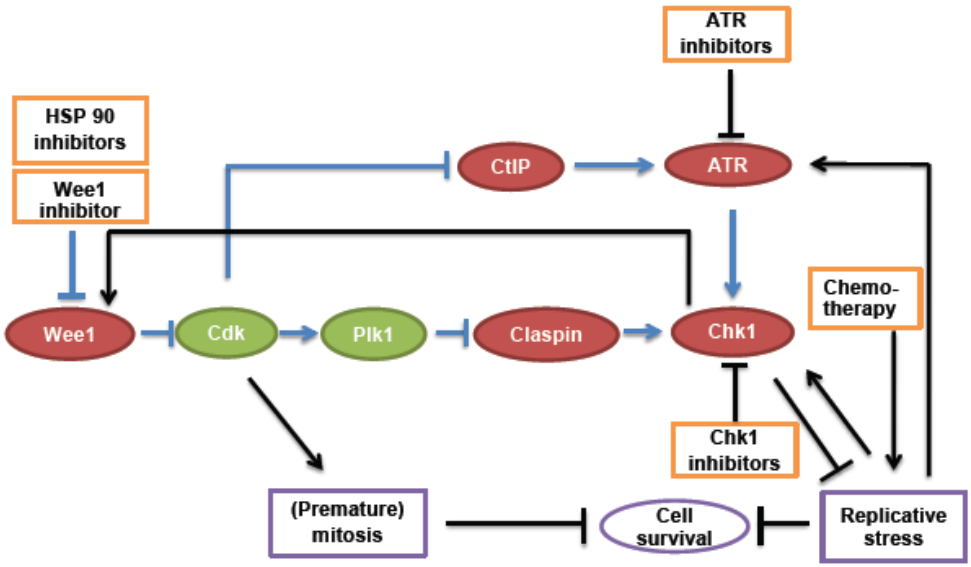


Figure IV.1: Molecular communication between Wee1 and Chk1. Wee1 inhibition can be achieved by direct small molecule inhibitors, or otherwise by HSP90 inhibition (Aligue et al. 1994). Chk1 and ATR are subject to similar inhibition strategies. Kinase activities mediate signaling cross-talk as depicted. Blue arrows indicate pathways investigated in this new study, black arrows refer to literature available (Figure was taken from the editorial Saini et al. 2015a).

IV.3 Pharmacological activation of p53 offers protection against the highly potent Wee1i-gemcitabine combination

Chemotherapies are known to produce various unwanted side effects. These include depletion of normal stem cell populations in blood, gut epithelia and hair (Galmarini et al. 2002). For instance, the nucleoside analogue gemcitabine frequently causes myelosuppression (Fossella et al. 1997). How can we improve this situation and protect non-cancerous cells from chemotherapy induced collateral damage? One approach might be the exploitation of the naturally occurring cell cycle checkpoint activation and DNA damage sensing machinery. It is commonly known, that p53 is a potent regulator of the cell cycle upon DNA damage, and that it is frequently mutated in cancers (Nigro et al. 1989). The drug Nutlin-3a is able to activate normal, but not mutant p53 (Coll-Mulet et al. 2006). This might be utilized to protect normal cells from chemotherapy-induced cellular toxicity. And indeed, previous reports have shown, that Nutlin-3a is able to protect p53 proficient cells from gemcitabine issued toxic effects (Kranz and Dobbelstein 2006). p53 activation through Nutlin-3a strongly induces its target gene p21, which halts the cell cycle at the G1/S transition (Polager und Ginsberg 2009) and therefore prevents cytotoxic effects of S phase chemotherapeutics. As we have used a Wee1 inhibitor and gemcitabine in combination, with both drugs being most active during S phase, our cells were protected in a p53 dependent manner when pretreated with Nutlin-3a (see Figure III.9).

According to our model, MDM2 antagonists might find new clinical applications as protective drugs. Chemical activation of cell cycle checkpoints in normal cells might help to reduce chemotherapeutic side effects in patients and could also further allow an increase of the total chemotherapeutic drug concentration applied.

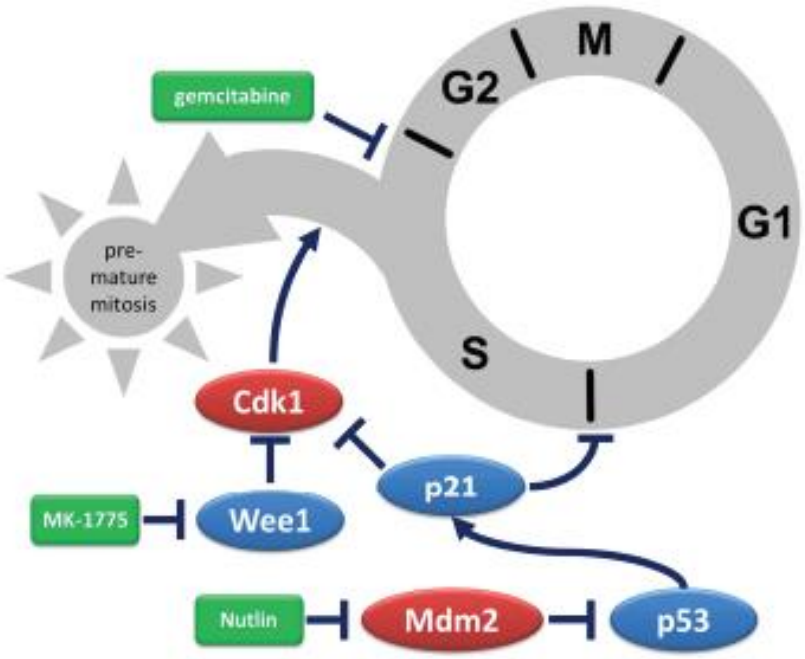


Figure IV.2: Summary of protective mechanisms triggered by MDM2 inhibition upon Wee1 inhibition. p53 stabilization via Nutlin-3a inhibits the G1/S phase transition by inhibiting Cdk1 through p21. This effect is able to protect p53 proficient cells against the Wee1 inhibitor MK-1775, as this inhibition normally hyper-activates Cdk1 and forces progression through both S and M phase, leading to premature mitosis. In this scheme, activators of cell cycle progression are depicted in red, inhibitors of cell cycle progression in blue, and drugs in green. Arrows indicate activation, lines that end with a bar indicate inhibition. The figure concept was drawn by Prof. Dr. Matthias Dobbelstein (from Li et al. 2015).

IV.4 Can nutlins still find their intended broad applications in clinics?

The engineering of nutlins and other MDM2 antagonizing drugs thrilled the cancer research community, the finding was termed the “awakening of the guardian angel” (Brown et al. 2009), and it did seem straightforward, cancer cells would stop proliferating and go into apoptosis upon the reactivation or overactivation of the p53 pathway. And indeed, in preclinical trials, this promise seemed to hold true, Nutlin-3a did induce cell death in both *in vitro* wildtype p53 cell lines and *in vivo* mouse xenograft experiments (Vassilev et al. 2004), with cell lines overexpressing MDM2 being the most sensitive to the drug. However, once the way was cleared for clinical patient trials, the results were more than disappointing: Liposarcoma patients were selected for the initial MDM2 antagonist clinical trial, as this tumor entity possesses wildtype p53 and amplified MDM2 expression (Momand et al. 1998), in theory a very suitable molecular situation for a MDM2 antagonist treatment approach. But in reality the study was not successful, as only 1 patient (out of 20) showed a partial response, with 14 showing stable disease and 5 remaining patients with progressive disease (Ray-Coquard et al. 2012). Furthermore, the patients were tormented with heavy side effects of the drug, such as thrombocytopenia and neutropenia. These adverse effects were not predicted from the preclinical studies and could have various causes, such as the p53-dependent activation of apoptotic markers NOXA and PUMA in the depleted blood cell populations. To explain these phenomena molecularly, MDM2 antagonist function needs to be investigated more intensively in normal tissues and animal experiments could be extended to non-human primates (Khoo et al. 2014). Taken together, Nutlin-3a seems to be a weak drug, as it induces cell cycle arrest reversibly in cell lines such as colon cancer cells (Paris et al. 2008), but not apoptosis (Rigatti et al. 2012). These observations imply that the amount of p53 accumulation needs to surpass the threshold from cell cycle arrest towards apoptosis induction, pushing the concentrations applied into the toxic segment, in which severe side effects arise. Taking this into consideration, MDM2 antagonists, as a single agent treatment against cancer, have failed in clinics.

IV.4.1 Hyper-activation of p53 through Nutlin-3a – Wip1 inhibitor combinatory treatment

So how can we still utilize Nutlin-3a as a chemotherapeutic drug? One possibility is the combination treatment with an agent, which also enhances p53 pathway functionality synergistically. As MDM2 antagonists have been shown to be cytotoxic substances in clinical trials, it might represent a concentration limiting constant in a combination therapy setup. Synergistic effects on apoptosis induction in various cell lines have been observed upon combining Nutlin-3a with Cdk inhibitors (Cheok et al. 2007) and thus there is the possibility, that Nutlin-3a reveals its full clinical potential when given in a combination with another synergistic drug.

In one of our other projects we have shown, that the inhibition of Wip1, a phosphatase that is induced by and inactivates p53 (Fiscella et al. 1997), through the allosteric small-molecule inhibitor GSK2830371 (Gilmartin et al. 2014), acts synergistically with Nutlin-3a to induce cell cycle arrest and senescence through strong induction of p21 (Sriraman et al. 2016). Independent from our findings, two other research groups have shown the same drug combination to act synergistically on induction of apoptosis in MDM2 overexpressing cell lines (Esfandiari et al. 2016; Pechackova et al. 2016). Taken together, these findings present a potent p53-pathway-activating drug combination, which might be suitable for clinical trials.

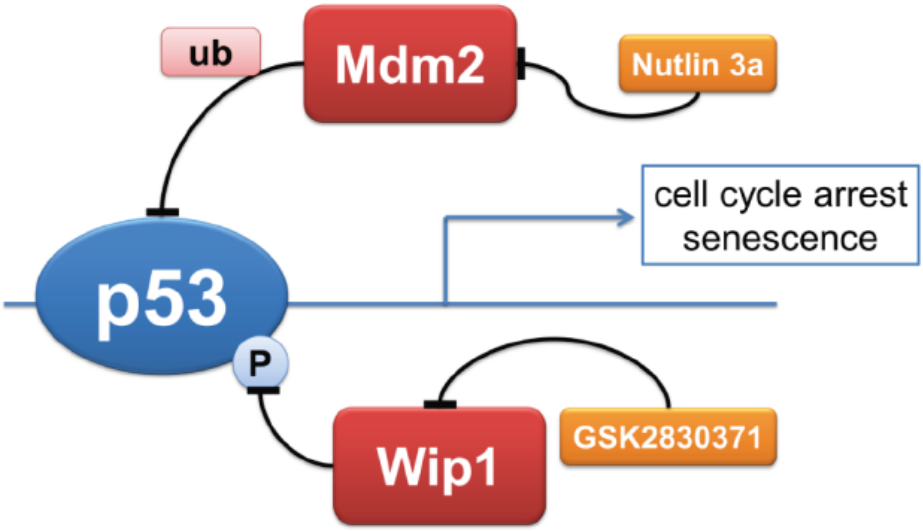


Figure IV.3: Potentiation of p53 activity through Mdm2 and Wip1 inhibition. p53 receives negative feedback from both Mdm2 and Wip1. When both feedback regulators are targeted by drugs simultaneously, p53 activity is enhanced to a greater extent than with each drug alone. As a result, the cells undergo sustainable cell cycle arrest and/or senescence. The figure concept was drawn by Prof. Dr. Matthias Dobbelstein (from Sriraman et al. 2016).

IV.4.2 The concept of cyclotherapy

The concept of cyclotherapy implies the protection of normal tissues, but not of malignant cells, from a cytotoxic drug, i.e. focusing toxicity on cycling cells, but not on cells that were arrested in the cell cycle. This approach aims at the reduction of side effects with intensification of the cytotoxic effects on the cancerous tissue. The main idea is to exploit the deficiency of cancer cells of certain cell cycle checkpoints: While the normal cell arrests upon chemical stimulation, the malignant cell continues to move into the chemotherapeutic sensitive cell phase and is hit with a lethal dose of the cytotoxic drug. Some possibilities to achieve cell cycle arrest include targeting of Cdks, growth factor starvation, and, of course, activation of p53 (reviewed in Blagosklonny und Pardee 2001; van Leeuwen 2012). The p53 inhibitor Nutlin-3a has already been tested for this purpose: Arresting the cell cycle at the G1/S phase in a p53 dependent manner protects the arrested cell from chemotherapeutic agents active in other cell cycle phases (van Leeuwen et al. 2012). This might have further importance for avoiding secondary cancers induced by chemotherapies (Boffetta and Kaldor

1994) or even radiation therapies (Ng and Shuryak 2014), as they are induced by the collateral damage of the primary tumor treatment. Different studies have reported pretreatment with Nutlin-3a to protect p53 proficient cells from both M-phase (Carvajal et al. 2005) and S-phase chemotherapeutic drugs (Kranz and Dobbelstein 2006; Li et al. 2015). Utilizing the p53 pathway as a molecular brake for cytoprotection of non-transformed and p53 wildtype cells seems an attractive idea to be implied in clinical cancer treatment, as it provides an approach to distinguish cancerous p53 loss of function from non-cancerous wild-type p53 cells. This distinction with p53 status as a biomarker has already been demonstrated in cell culture experiments (van Leeuwen et al. 2012), and most interestingly, Nutlin-3a pretreatment has already been shown to reduce toxic side effects of a Plk1 inhibitor *in vivo*, without affecting its anti-cancer effects (Sur et al. 2009). Intensifying the molecular Nutlin-3a brake by boosting the p53 pathway, for instance by simultaneous inhibition of the Wip1 phosphatase (Sriraman et al. 2016), might add to the potency of the cyclotherapy approach and furthermore give a renaissance to p53 pathway modifying drugs in clinics.

Until today, oncologists struggle to make a difference between malignant and untransformed tissues in most cancer types, commonly causing collateral damage by applying classical chemotherapeutic drugs to the patient, being unable to specifically target the malignant tissue of the disease. Personalized medicine, through introduction of highly sophisticated molecular diagnostic technologies into clinics, will try to unravel single specific weaknesses of the unique cancer in the unique patient. This is the promise of the cancer patient care of tomorrow. Already today, specific targeting of a unique molecular abnormality in CML via Imatinib (Roskoski 2015) has provided millions of patients with a long and high quality life. Cancer can be contained, but we have to take the clinics to the molecular big data level. It is time to mine the –omics era for the cancer answer.

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MK-1775|AZD-1775;AZD1775;AZD 1775|CAS 955365-80-7 Buy MK-1775 from supplier medchemexpress.com. http://www.medchemexpress.com/MK-1775.html?gclid=CjwKEAjw5cG8BRDQj_CNh9nwxTUSJAAHdX3fMuzgsrG2Qk1m_EkGT7mmFbnZIk60nizNmUYjm796wxoCpizw_wcB; Accessed on 21.07.2016

VI Supplement

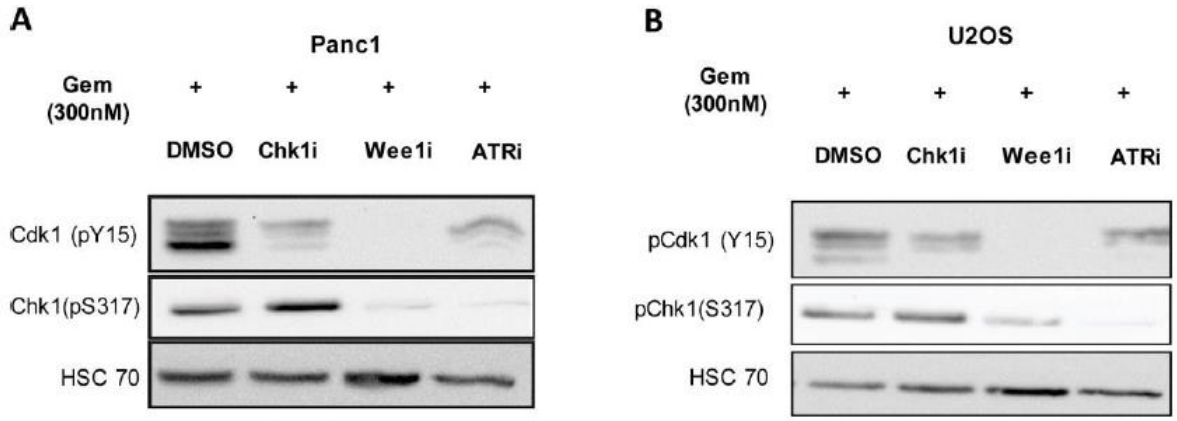


Figure S1: Wee1 inhibition prevents phosphorylation of Cdk1 at Tyr15.
The Wee1 inhibitor MK-1775 (0,5µM), but not Chk1 (2,5µM) or ATR (5µM) inhibitors prevent phosphorylation of Cdk1 at Tyr15 after 24h of treatment in both Panc1 and U2OS cells. This phosphorylation site has been previously described and suggests the specific functionality of the Wee1 MK-1775 inhibitor. Experiments were performed by Dr. Priyanka Saini.

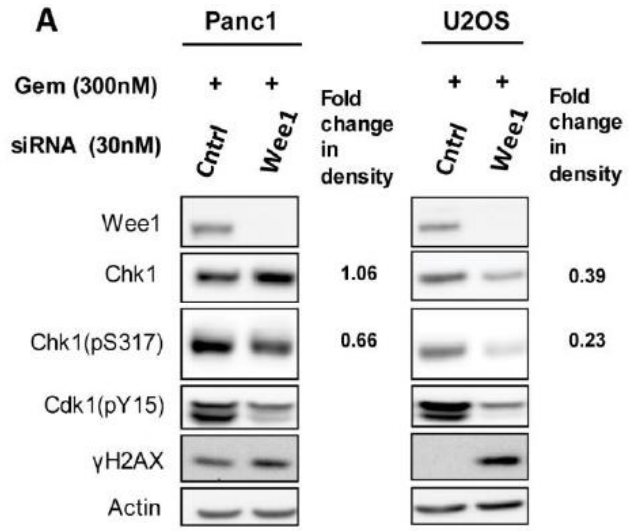


Figure S2: Wee1 siRNA knockdown prevents phosphorylation of Cdk1 at Tyr15.
In both U2OS and Panc1 cell lines, reduction of the Wee1 protein via siRNA mediated knockdown for 48h also reduced the phosphorylation of Cdk1 at Tyr15 upon a subsequent treatment with 300nM gemcitabine, further providing evidence for the functionality of the Wee1 inhibitor MK-1775 compound. The experiment was performed by Dr. Priyanka Saini.

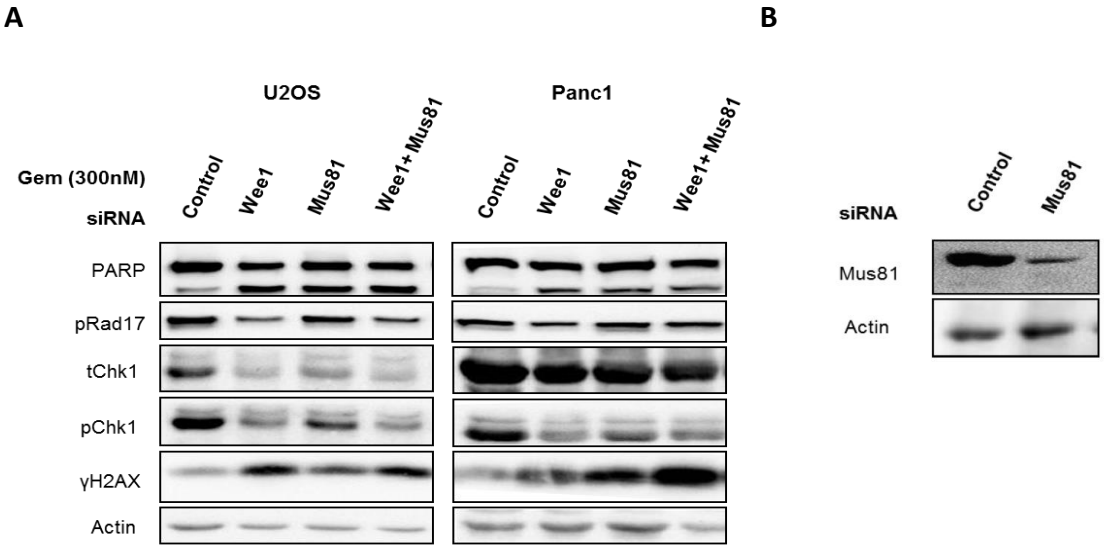


Figure S3: Wee1 knockdown induced p-Chk1 reduction and yH2AX elevation is independent of Mus81. (A) In both U2OS and Panc1 cell lines, reduction of the Wee1 protein via siRNA mediated knockdown for 48h reduced Chk1 phosphorylation and induced accumulation of yH2AX upon a subsequent treatment with 300nM gemcitabine. This effect has been shown to be independent from Mus81 function. (B) A siRNA knockdown with Mus81 was performed. After 48h, cells were harvested and processed for western blot analysis which shows functionality of the Mus81 siRNA. The experiments were performed by Dr. Priyanka Saini.

Acknowledgements

First and foremost I have to thank Prof. Dr. Matthias Dobbelstein for everything he has done for me. As my, in German, “Doktorvater”, he has supported me with guidance, possibilities and living expenses. I am very thankful that our ways have crossed here in Göttingen.

Secondly, I want to thank Dr. Priyanka Saini for the introduction into Molecular Oncology. She has also treated me like family, being patient, kind and supportive at the same time. With her teaching, which was not just professional but also caring, I am now able to move freely in our research field. Priyanka, I wish you well and hope, that life will treat you as kind as you treat others.

A big thank you also goes to Anusha Sriraman, who has done a tremendous job on the revision of our paper, when I was busy with my first medical state examination.

Many thanks also go to the entire group of Molecular Oncology, which is maintaining a clean and productive infrastructure, as well as a communicative and helpful scientific environment.

I want to thank Prof. Dr. Peter Jochimsen for showing me that there are no borders if you can imagine beyond and that one should not fear, as long as one can dream. Since 2007, he has shown presence in crucial moments of my life and I am very thankful that he has been saved from cancer to guide me through my most difficult times.

VII Deutsche Zusammenfassung

Die Kombination Wee1 Inhibitor MK-1775 und Gemcitabin kann sowohl Krebszellen *in vitro*, als auch Xenografts in Mäusen effizient abtöten, aber der molekulare Mechanismus dieser wirkungsvollen Kombination wurde bislang nicht vollständig aufgeklärt. Unsere Ergebnisse zeigen, daß MK-1775 nicht nur die Wee1 Aktivität blockiert, sondern zudem die Aktivierung des ATR / Chk1-Signalwegs in Gemcitabin-behandelten Zellen in einer Cdk1-abhängigen Weise reduziert. Diese Ergebnisse legen nahe, dass Wee1 Inhibitoren nicht nur dazu in der Lage sind, Zellzyklus-Checkpoints zu übergehen, sondern zudem auch replikativen Stress erhöhen und die Chemosensitivität gegenüber Nukleosidanaloga durch Reduktion der DNA damage response verstärken. Jedoch limitiert die erhebliche Toxizität von MK-1775 sowohl in präklinischen als auch in klinischen Studien seinen Anwendungsbereich stark.

Mehr als 50% aller malignen Tumoren tragen eine Mutation im TP53-Gen. Wir konnten unter Verwendung des p53-MDM2-Antagonisten Nutlin-3a einen selektiven Schutz für p53-Wildtyp-Zellen gegen die zytotoxischen Wirkungen von Wee1-Inhibitoren herstellen. Die Vorbehandlung von p53-Wildtyp-Zellen mit Nutlin-3a bewirkt eine transiente Arretierung im Zellzyklus bei G1/S. Nutlin-3a-vorbehandelte, transient arretierte Zellen zeigen ein verbessertes Überleben gegenüber der Kombination aus dem Wee1-Inhibitor MK- 1775 und Gemcitabin. Die Nutlin-3a-Vorbehandlung reduziert sowohl die DNA damage response als auch die Caspasen-Aktivierung in einer p53-abhängigen Weise. MDM2-Antagonisten können daher selektiv p53-kompetente Zellen gegen die zytotoxischen Wirkungen von Wee1-Inhibitoren schützen, insbesondere wenn sie mit einer S-phasenspezifischen Substanz wie dem Nukleosidanalogon Gemcitabin kombiniert werden. Dieser Ansatz könnte helfen, Nebenwirkungen von Wee1-Hemmstoffen in der klinischen Anwendung für Patienten zu verringern oder ganz zu vermeiden.

Curriculum Vitae

My name is Yizhu Li, I was born on the 6th of July 1987 in Shanghai, China. I am the first born son of the business woman Wei Qian and the engineer Gang Li.

My family moved to Kiel, Germany, in 1991. I was soon enrolled in the elementary school Claus-Rixen Schule, Altenholz. After completion of my elementary education in 1996, I was a student at the Humboldt-Gymnasium, Kiel. In between my school career, I was an exchange student at the Miller Highschool, Corning/Ohio/USA in 2003/04. In 2006 I received my Abitur with a final grade of 2,1.

After my school education, I went to the German Navy for nine months until March 2007. Afterwards I went to China for five months to study Chinese. Here I witnessed the cancer diagnosis of my grandmother and a strong desire to study medicine arose within me.

But, unable to study medicine due to my grade point average, I started biology studies at Kiel University, as biology was my favorite subject in school. I was subsequently rewarded my Bachelor of Science in biology in July 2010, with the final score of 1,4. Following into the same direction as my bachelor thesis, I have decided to intensify my knowledge in the field of developmental biology. Therefore, I enrolled into the Molecular Biosciences program at the University of Heidelberg with a major in developmental biology. Overcoming various difficulties during my master studies, I was awarded my Master of Science degree in June 2013, with the final score of 1,3.

I started my medical studies at the University of Göttingen in October 2013. I have passed my first medical state exam with a grade of 2,0, and I am currently in the clinical part of my medical education. I want to specialize in inner medicine and hope to undergo my expert training in the hematology/oncology field.

Due to great support from Prof. Dr. Matthias Dobbelstein, I have rediscovered my passion for science. I wish to continue scientific work, if possible as a PhD student, to one day conduct translational research and to help cancer patients benefit from the innovation generated in laboratories.

Dear commission members,

Many thanks for your efforts to evaluate my thesis entitled **"Inhibition of the kinase Wee1 - Cytotoxic mechanisms and autoprotection by the tumor suppressor p53".**

I was informed that the reproducibility of my experiments, and especially of some results obtained by immunoblot analyses, were questioned by some of the reviewers. Therefore, please find attached an addendum. This supplementary material is supposed to confirm the observations described in the Western Blots of my thesis.

Before describing this material, let me also draw your attention to the fact that the majority of my findings have been peer-reviewed, revised and published in a well-renowned journal. Thus, at least our reviewers deemed the quality of our experiments and results as being sufficient to support our conclusions.

Moreover, some of the experiments were carried out in different cell lines, derived from different cancer entities, and the results were all supporting the same biological model. This further argues in favor of the reproducibility of our results. In most figures of this addendum, both the osteosarcoma derived U2OS cell line and the pancreatic cancer derived Panc1 cell line have been evaluated in side by side experiments.

Furthermore, a time course experiment was presented, which shows the accumulation of DNA damage in a yH2AX readout and the induction of apoptosis through a PARP cleavage readout from 4 to 24 hours (see Figure 1.1). Finding the same tendency at different time points, albeit to different extent, also argues that the results are reproducible.

Despite these considerations, we do appreciate the demand of the committee for further confirmation of the results. The following addendum contains a collection of immunoblot analyses that correspond to figures in my thesis. Whenever possible, densitometry measurements were made for the DNA damage indicator proteins phospho-Chk1 (pChk1) and yH2AX, in further support of our conclusions.

I hope this additional presentation of our data will help to convince the commission of its reproducibility.

With many thanks for your efforts in evaluating my thesis, and with my best regards,

Yizhu Li

Göttingen, 3rd of July 2017

Table of Contents

Cited reference:

Saini P, Li Y, Dobbelstein M (2015b): Wee1 is required to sustain ATR/Chk1 signaling upon replicative stress. Oncotarget 6, 13072–13087

Explanation for all following graphs: Figures from my thesis are marked with **red** outlines. The corresponding additional Western Blots and densitometry measurements can be found below the marked graphs.

1. Wee1 inhibition decreases Chk1 activation in Wee1i – Gemcitabine co-treated cells

1.1 Thesis Figure III.2 (p. 38)

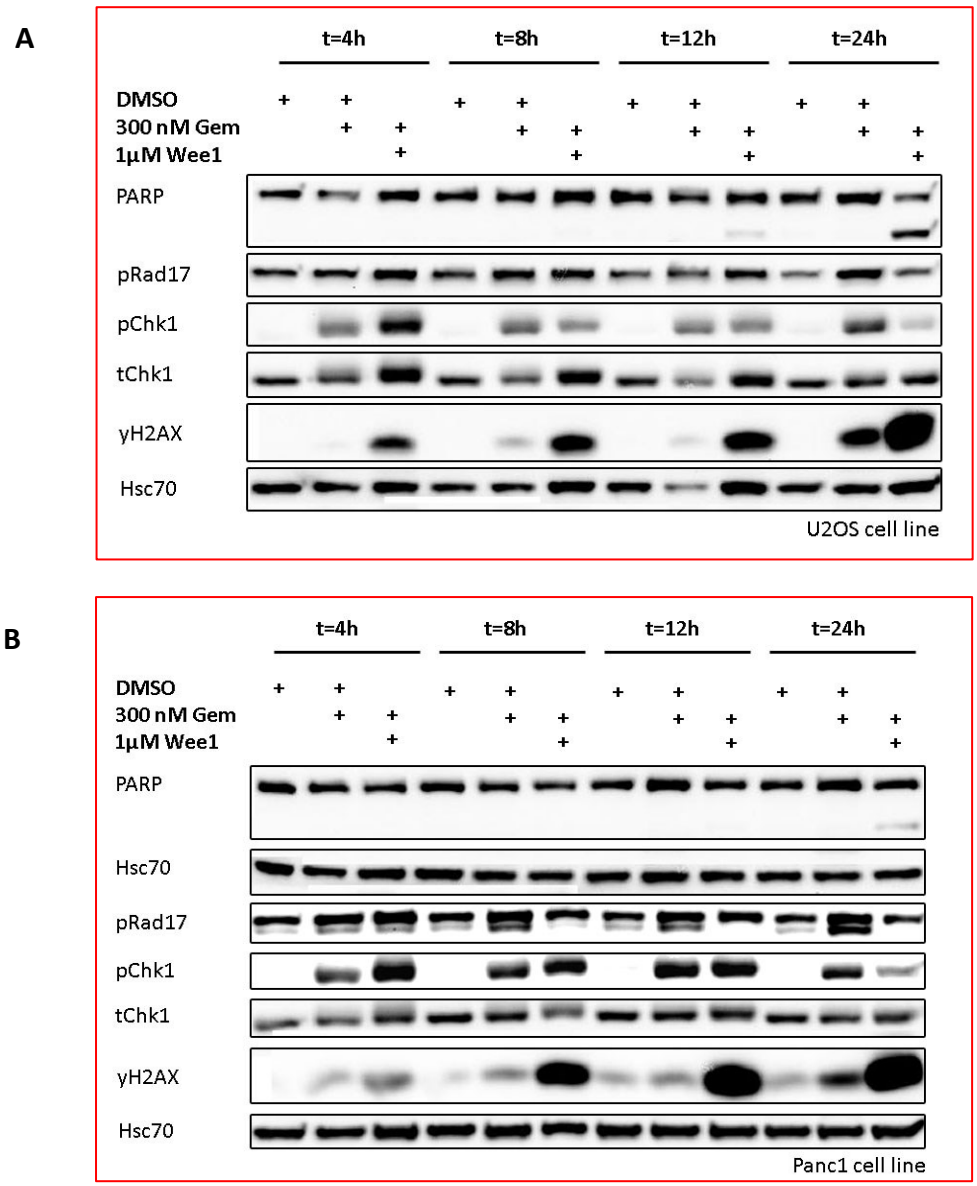


Figure 1.1: Wee1 inhibition decreases Chk1 activation in Wee1 inhibitor – Gemcitabine co-treated cells in a time dependent manner. U2OS and Panc1 cells were treated with Wee1 inhibitor (MK-1775) and Gemcitabine. Samples were harvested according to the time course experiment and prepared for Western Blotting.

1.2 Figure 2A and 2B from publication (Saini et al.,2015)

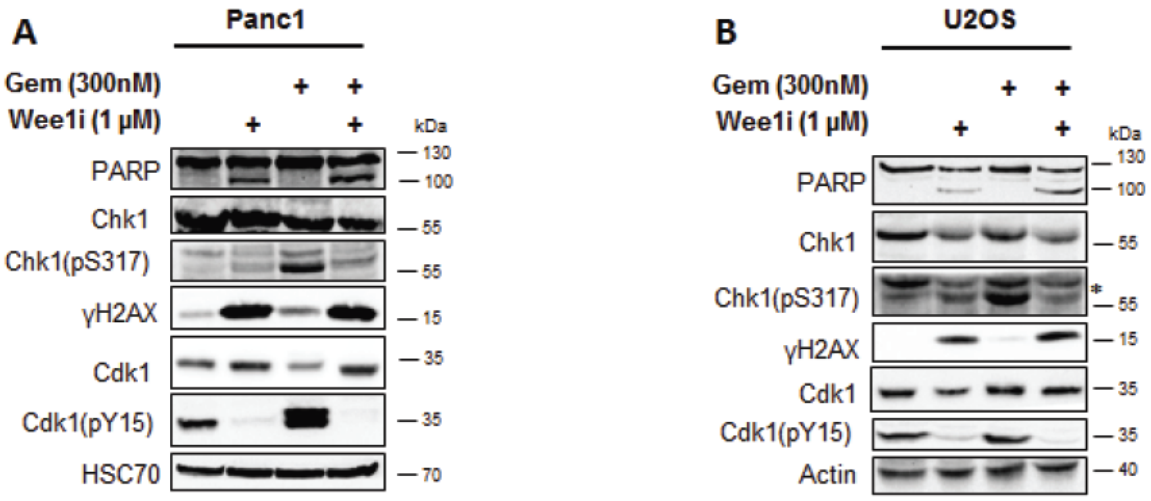


Figure 1.2: Inhibition of Wee1 decreases the phosphorylation of Chk1 in Gemcitabine-treated cells. A, B. Panc1 and U2OS cells were treated with 1μM Wee1i or DMSO, with or without 300nM Gemcitabine, for 24 h. Blots of cell lysates were stained. From Figure 2 from Saini et al., 2015.

The Figure 1.2 from Saini et al., 2015 corresponds to the 24h time point of Figure 1.1 from the thesis. The induction of PARP cleavage and γH2AX upon the co-treatment of Gemcitabine and Wee1i and the reduction of pChk1 upon Wee1i treatment can be clearly observed in both figures.

2. Decrease in Chk1 phosphorylation is independent of caspase activity

2.1 Thesis Figure III.3 (p. 39)

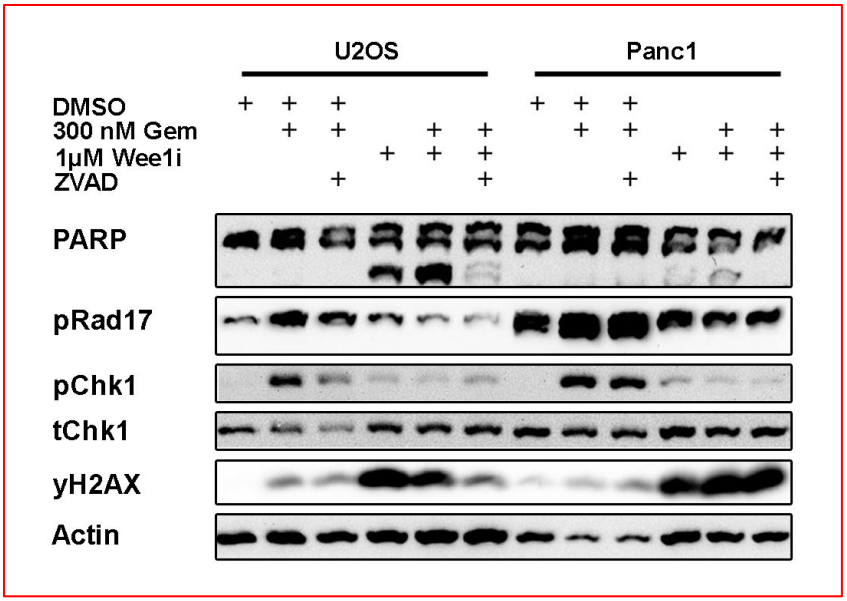


Figure 2.1: Decrease in Chk1 phosphorylation is independent of caspase activity. U2OS and Panc1 cells were treated with with Wee1 inhibitor (MK-1775) and Gemcitabine, with and without 20 µM of caspase inhibitor Z-VAD. The samples were harvested after 24 h and prepared for Western Blotting.

2.2 Figure 2E and 2F from publication (Saini et al., 2015)

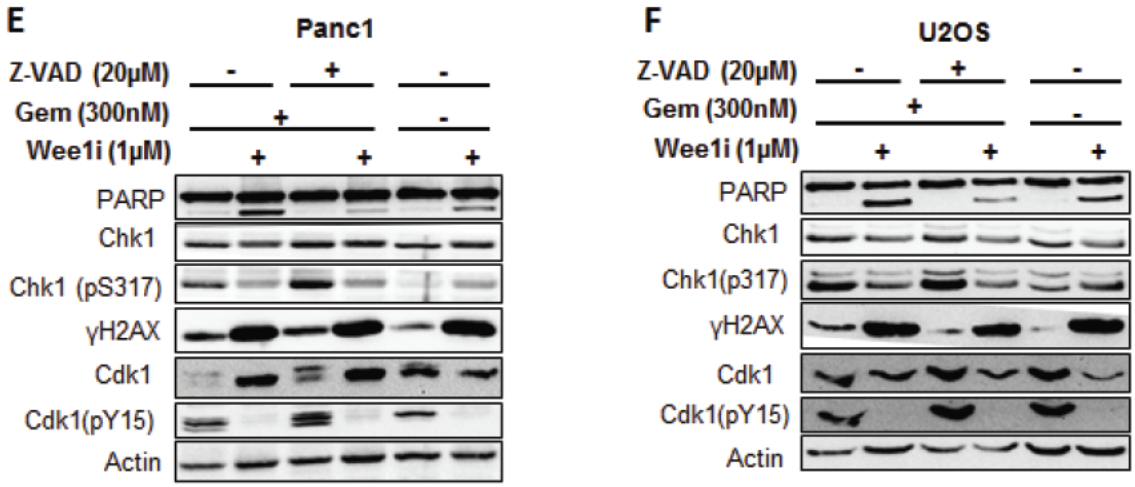


Figure 2.2: Inhibition of Wee1 decreases the phosphorylation of Chk1 in Gemcitabine-treated cells. Cells were treated with Wee1i or DMSO, with or without Gemcitabine, in the presence or absence of the pan-caspase inhibitor Z-VAD.fmk at the indicated concentrations. After 24 h, the cells were subjected to immunoblot analysis. From Figure 2 Saini et al., 2015.

Cells in this experimental setup (Figures 2.1 and 2.2) were treated with inhibitors for 24h. As the pChk1 expression pattern is not altered upon the caspase inhibitor ZVAD, we propose that dephosphorylation of Chk1 upon Wee1i treatment is independent from apoptosis.

3. Decrease in Chk1 phosphorylation in Wee1i treated cells is mediated by Cdk1

3.1 Western Blots

3.1.1 Thesis figure III.4 (p. 40)

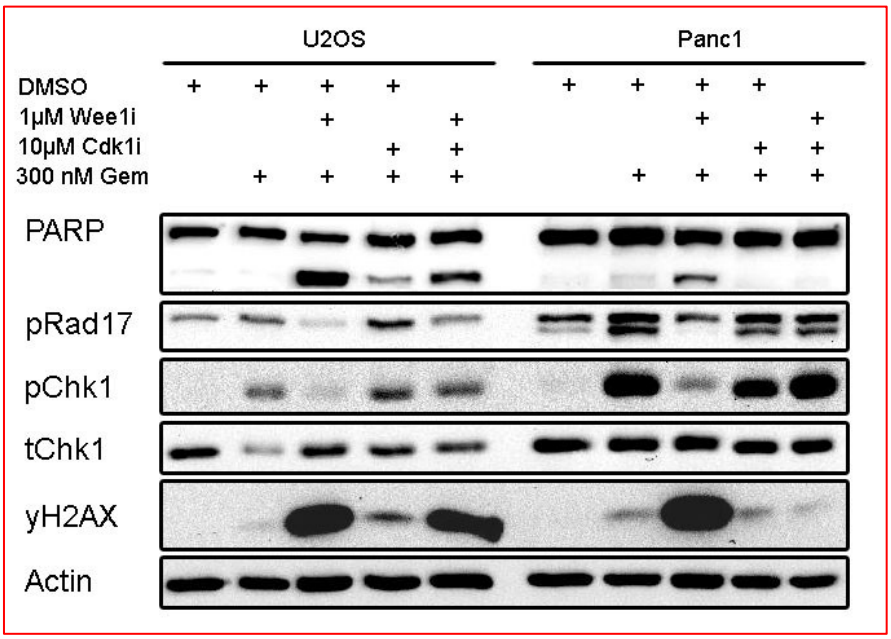


Figure 3.1: Decrease in Chk1 phosphorylation in Wee1i treated cells is mediated by Cdk1. Cells were treated with Cdk1 and Wee1 inhibitors in the presence of Gemcitabine. The samples were harvested after 24 h and prepared for western blotting.

Using the Figures 3.1-3 densitometry measurements for pChk1 and yH2AX were conducted. It can be observed in both U2OS and Panc1 cell lines that the co-incubation of Gemcitabine with a Wee1 inhibitor significantly decreases the pChk1 signal (Graphs 3.1). Furthermore, upon the addition of the Cdk1 inhibitor RO-3306 the suppression of the signal was rescued, and this effect was more prominent in the Panc1 cell line.

Co-treatment with Gemcitabine and Wee1i resulted in a synergistic accumulation of yH2AX signal in the densitometry measurements, which was rescued by additional treatment with a Cdk1 inhibitor (Graph 3.2). The effects were visible in both cell lines, but the decrease of the yH2AX signal through Cdk1i treatment was more prominent in the Panc1 cell line.

Taken together, there is an opposing correlation between the signals of yH2AX and phosphorylated Chk1 which can be manipulated by Cdk1 inhibition, suggesting its regulation by Cdk1 and emphasizing the S-phase DNA replication protective role of Chk1 in the DNA damage response. Furthermore, the Panc1 cell line seems to rely more on Chk1 for the DNA damage response than U2OS cells, this might be due to its mutant p53 status, therefore relying more on additional cell cycle checkpoint mechanisms.

3.1.2 Figure 4D from publication (Saini et al., 2015)

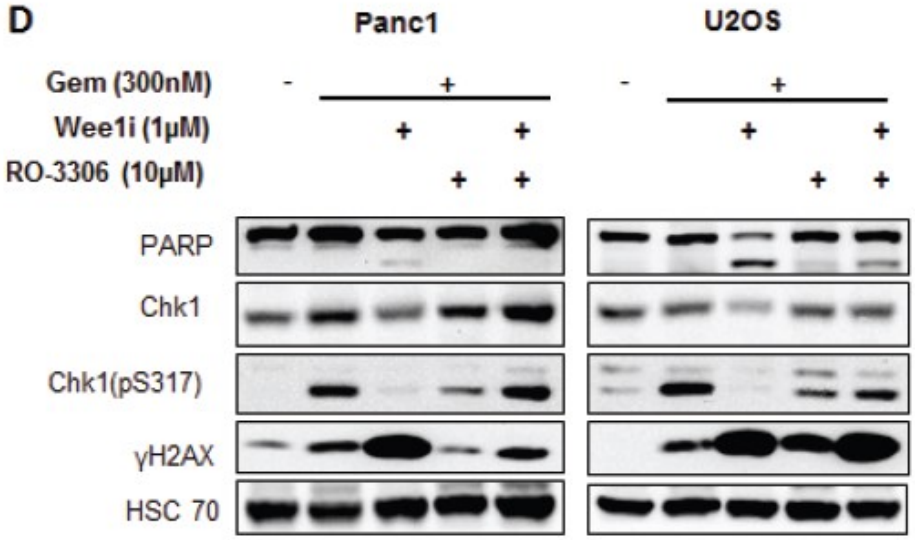


Figure 3.2: Cdks mediate the attenuation of the ATR-Chk1 pathway by Wee1 inhibition. Panc1 and U2OS cells were treated with Wee1 inhibitor (Wee1i) or DMSO, with or without Gemcitabine, in the presence or absence of RO-3306 (a Cdk1 inhibitor) at the indicated concentrations for 24 h. From Figure 4 Saini et al., 2015.

3.1.3 Additional Figure

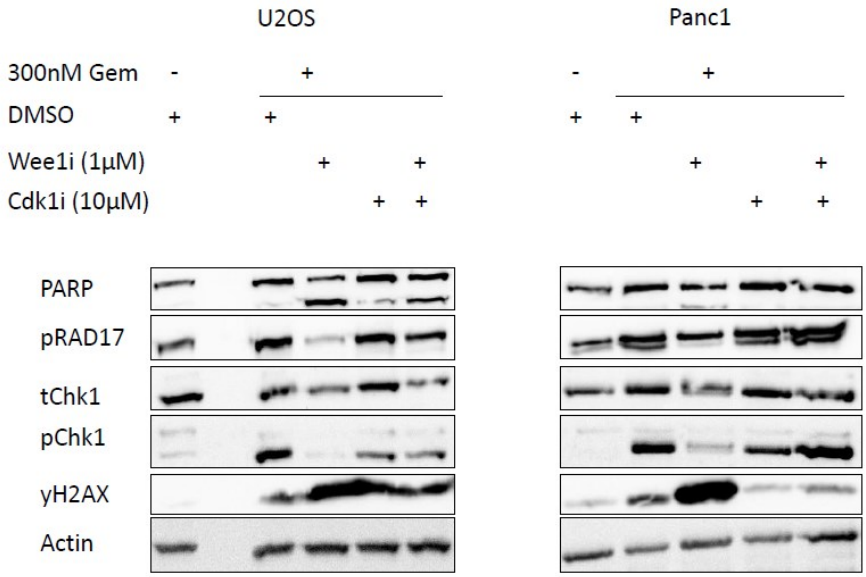


Figure 3.3: Cells were treated with Cdk1 and Wee1 inhibitors in the presence of Gemcitabine. The samples were harvested after 24 h and prepared for Western Blotting.

3.2 Densitometry measurements

3.2.1 Densitometry of the pChk1 signal from Western Blots

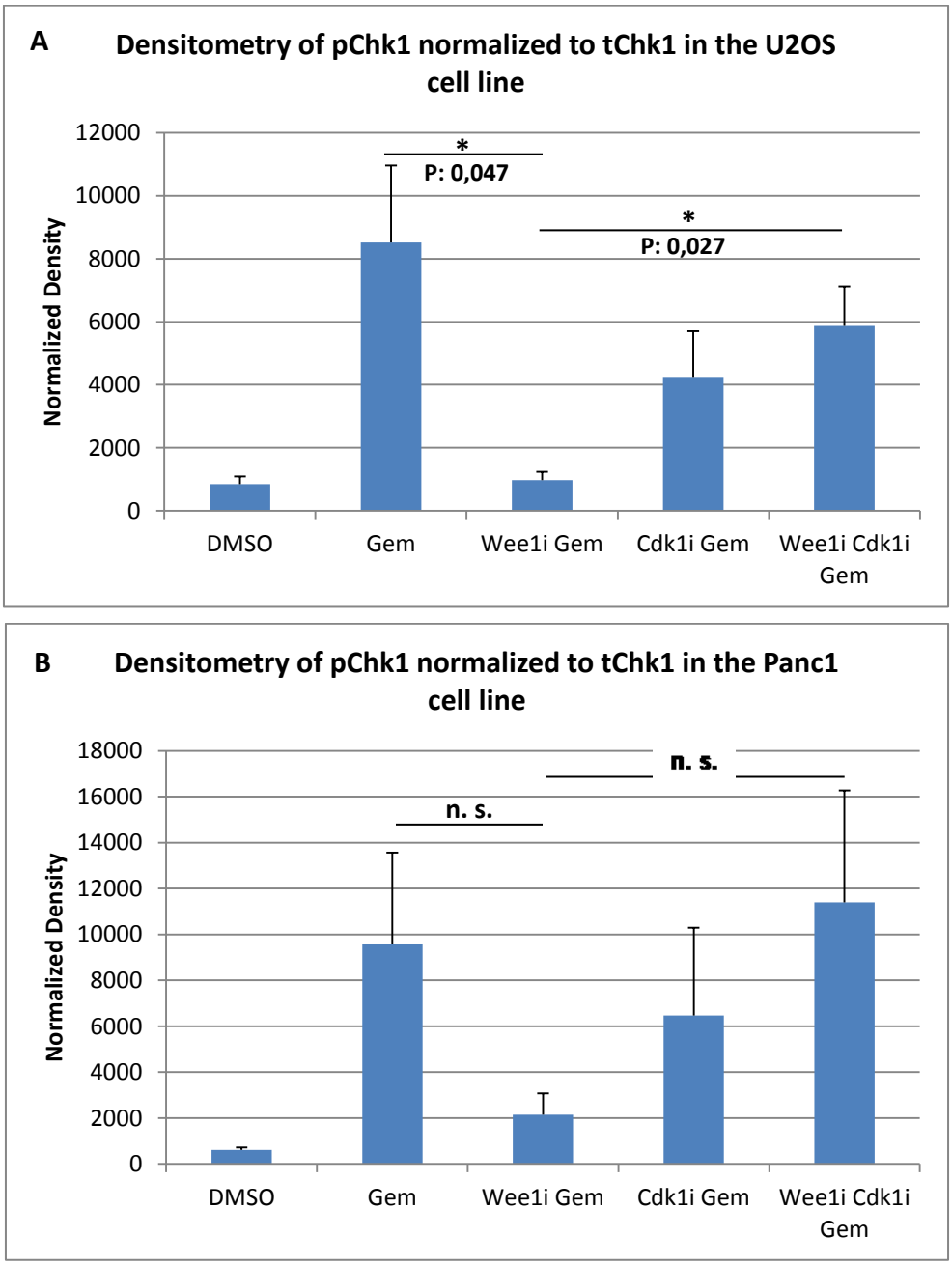


Table 3.1: Decrease in Chk1 phosphorylation in Wee1i treated cells is mediated by Cdk1. Gemcitabine induced pChk1 was significantly reduced with Wee1i co-treatment. This effect was reversed by further addition of a Cdk1 inhibitor (Cdk1i). Results in the Panc1 cell line were not significant in the densitometry measurement as the standard error was too high. Error bars represent the standard error (n=3). Significance was assessed by Student's t-test.

3.2.2 Densitometry of the yH2AX signal from Western Blots

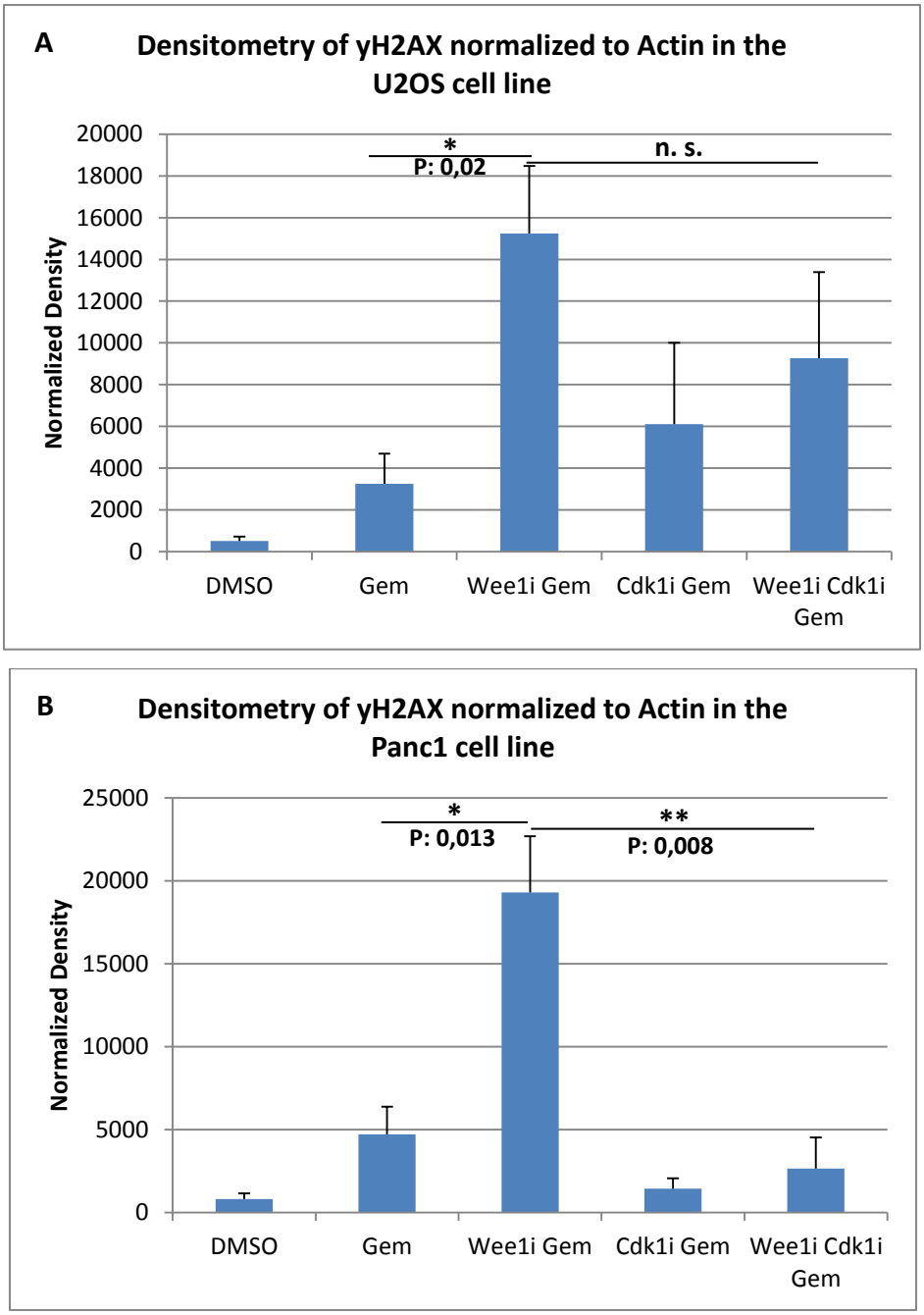


Table 3.2: Wee1i induced yH2AX accumulation with Gemcitabine co-treatment is rescued by Cdk1i addition. Co-incubation of Wee1i with Gemcitabine significantly increases yH2AX in both U2OS and Panc1 cell lines. This effect is rescued by addition of a Cdk1i. The observation is more prominent in the Panc1 cell line, this might be due to the stronger increase in pChk1 when compared to the U2OS cell line. Error bars represent the standard error (n=3). Significance was assessed by Student's t-test.

4. siRNA mediated knockdown of Cdk1 restores the phosphorylation of Chk1

4.1 Figure S2D and S2E from thesis (Saini et al., 2015) and thesis figure III.5 (p. 41)

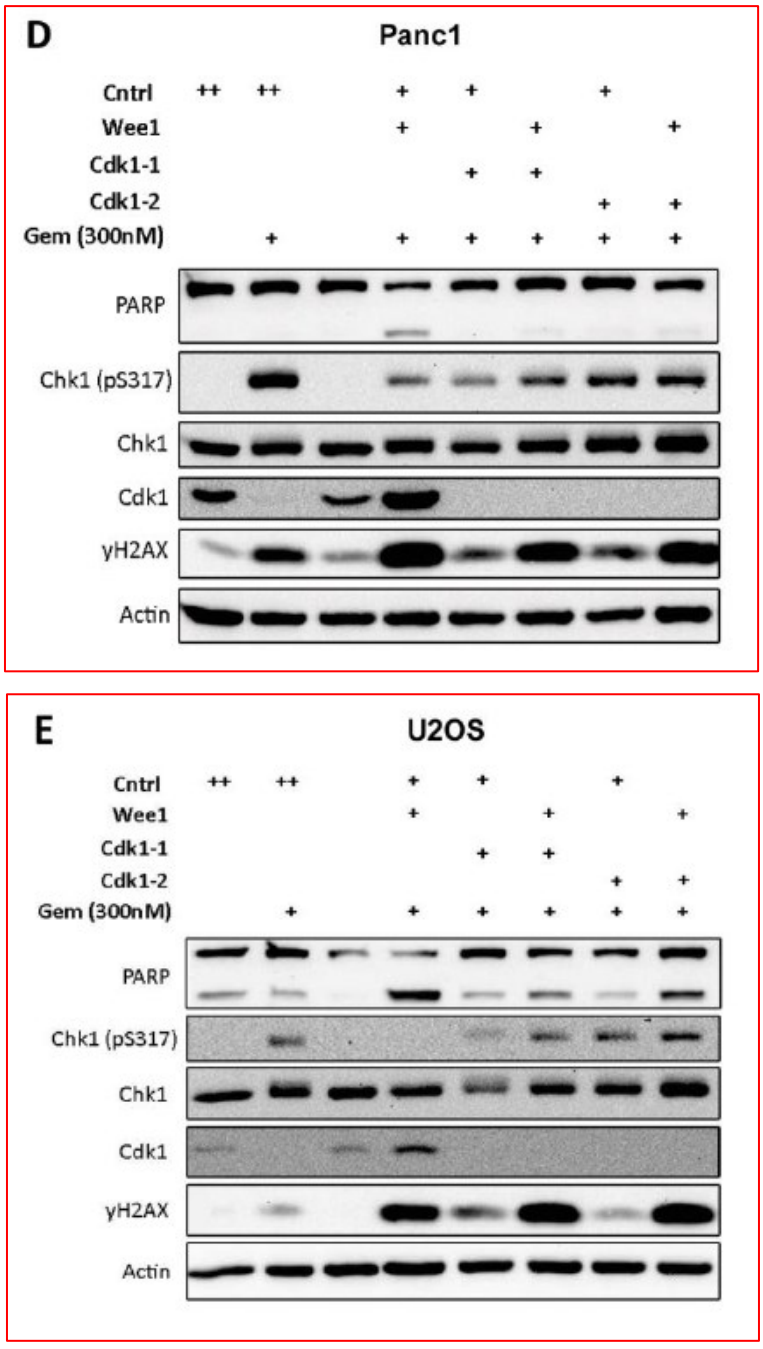
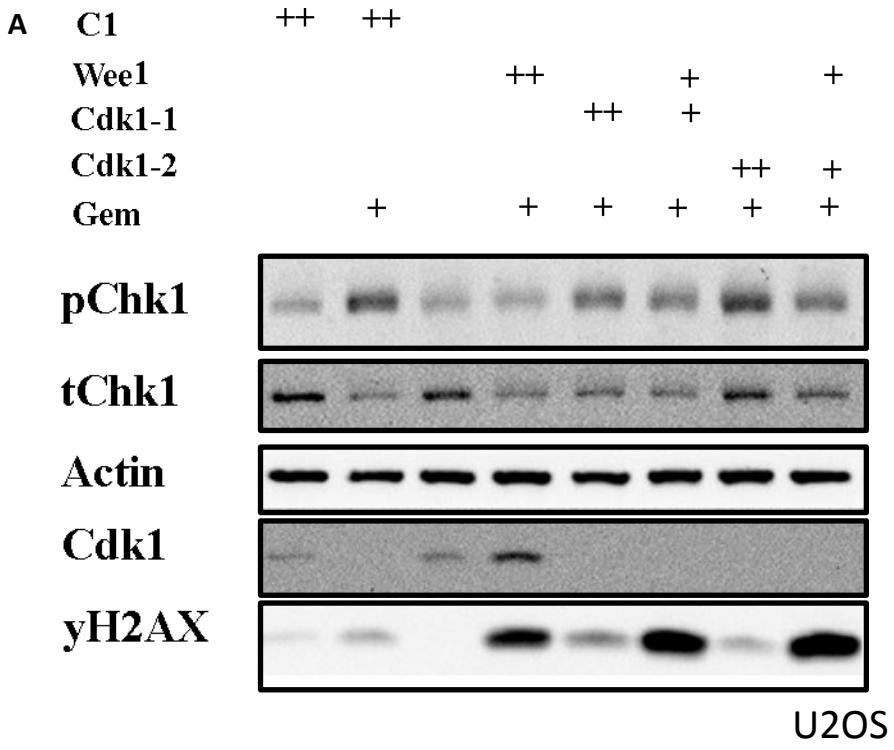
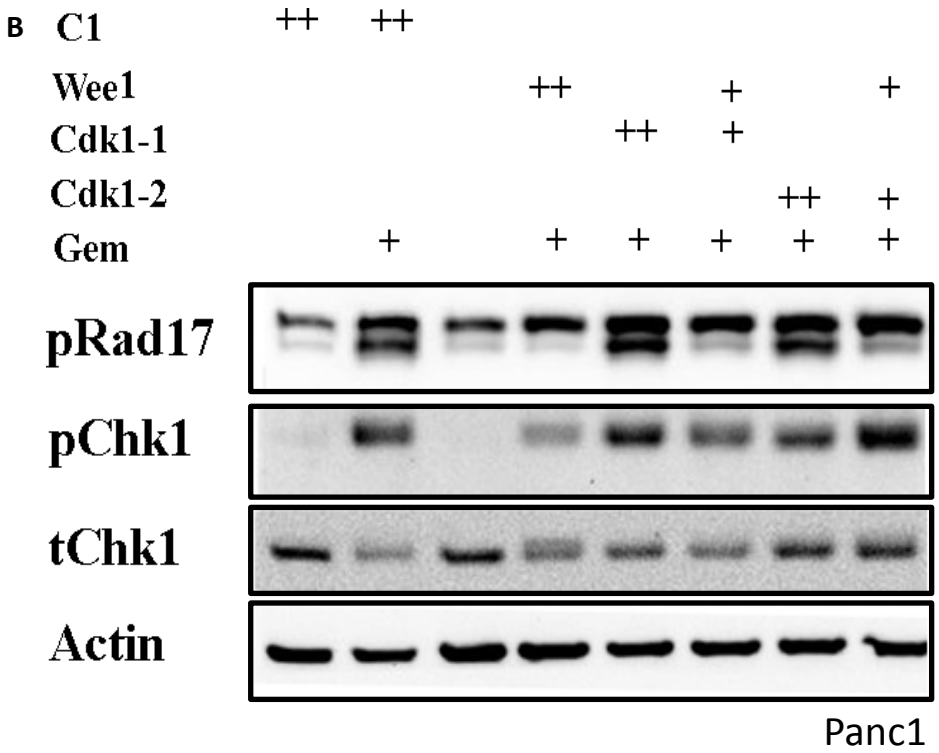


Figure 4.1: siRNA mediated knockdown of Cdk1 restores the phosphorylation of Chk1 upon simultaneous knockdown of Wee1 and treatment with Gemcitabine. 10nM of each siRNA (Cdk1, Wee1, scrambled) was used for transfecting cells. After 48 h of transfection, 300 nM Gemcitabine was added for another 24 h. The cells were then harvested and immunoblotted.

4.2 Additional figures





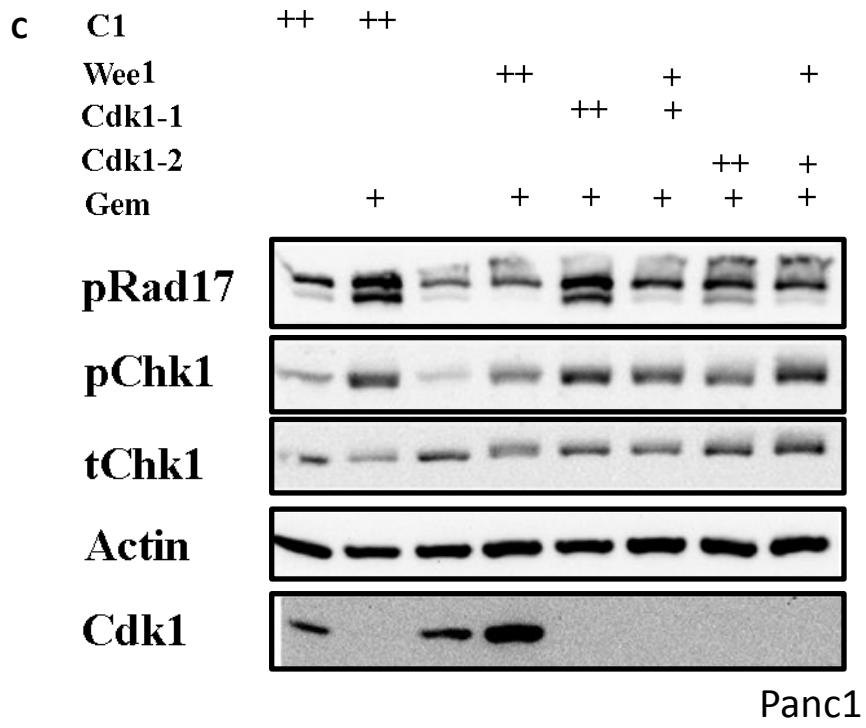


Figure 4.2: Corresponding figures to Figure 4.1.

4.3 Densitometry of the pChk1 signal from Western Blots.

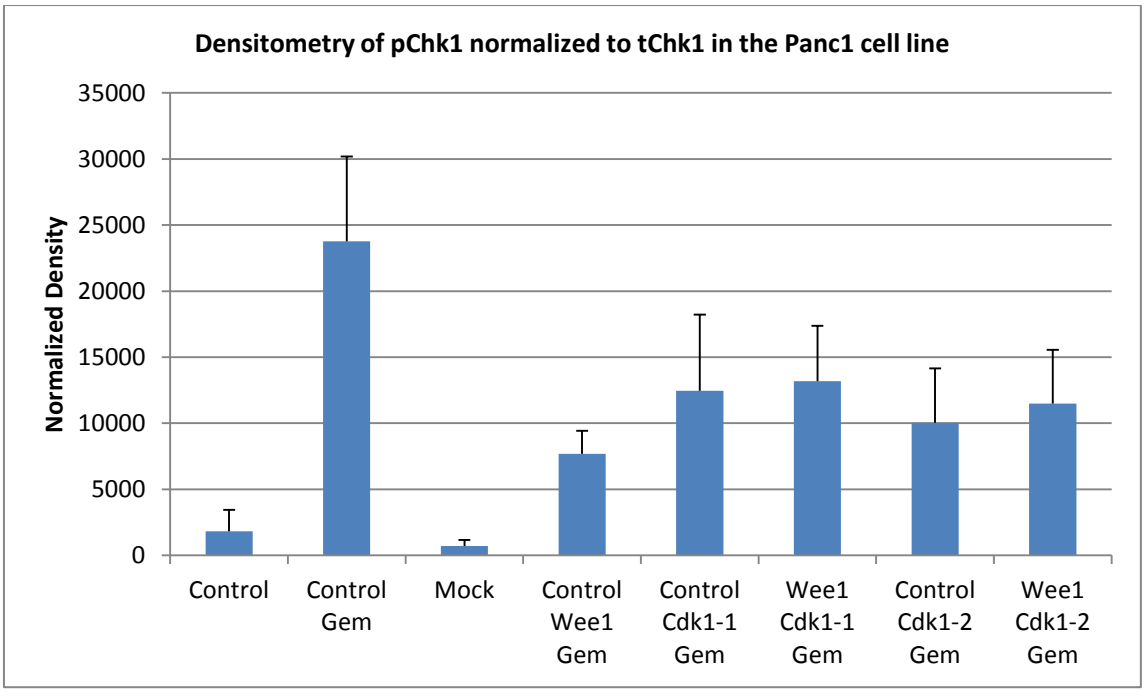


Table 4.1: siRNA mediated knockdown of Cdk1 restores the phosphorylation of Chk1 upon simultaneous knockdown of Wee1 and treatment with Gemcitabine. Gemcitabine induced pChk1 was strongly reduced by Wee1 knockdown, the pChk1 signal was rescued by co-knockdown of Cdk1. Error bars represent the standard error (n=3). The differences were not statistically significant, maybe due to the leakiness of siRNA mediated knockdowns.

5. Nutlin-3a protects cells against the Gemcitabine – Wee1 inhibitor co-treatment

A 24h Nutlin-3a pre-treatment is able to reduce Gemcitabine and Wee1i induced yH2AX, this is due to activation of the p53 depended G1/S cell cycle checkpoint, which protecting the cell to accumulate DNA damage in the sensitive S-phase. This rescuing effect was quantified by densitometry (n=3) and proved to be significant for the Wee1i and Wee1i / Gemcitabine conditions.

5.1 Thesis figure III.8 (p. 45)

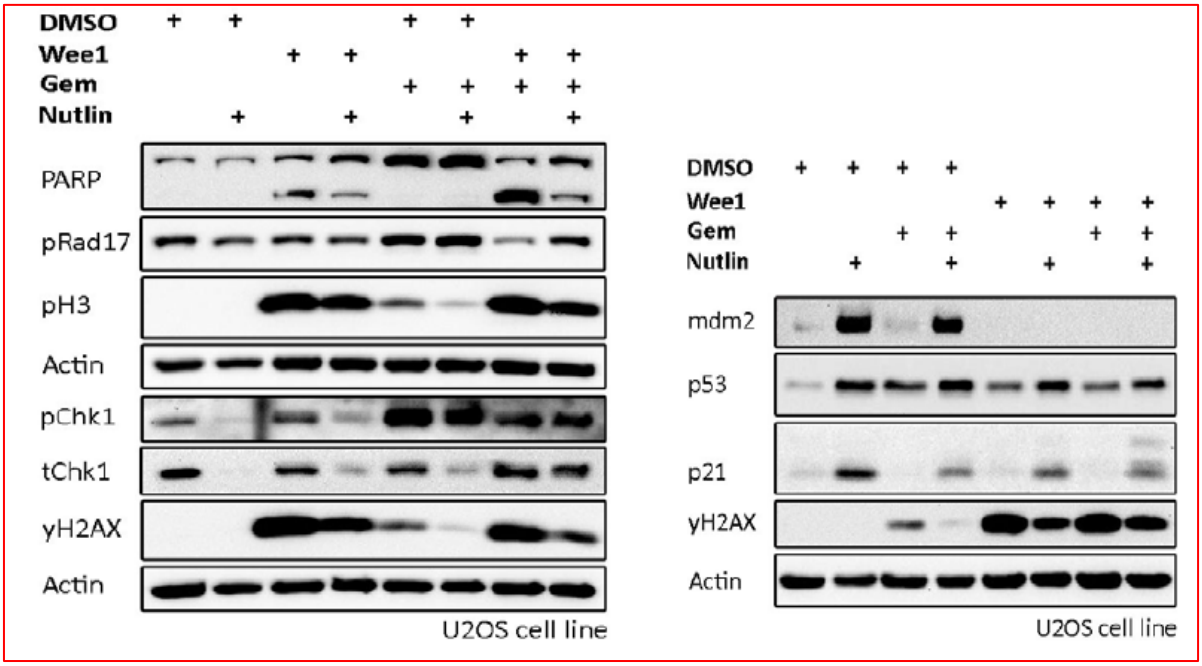


Figure 5.1: Nutlin-3a protects cells against the Gemcitabine – Wee1 inhibitor co-treatment. Cells were treated with Nutlin-3a (8µM) for 24h, followed by treatment with Wee1 inhibitor and Gemcitabine with Nutlin-3a for another 24h. The samples were then harvested and prepared for western blot analysis.

5.2 Additional Figure

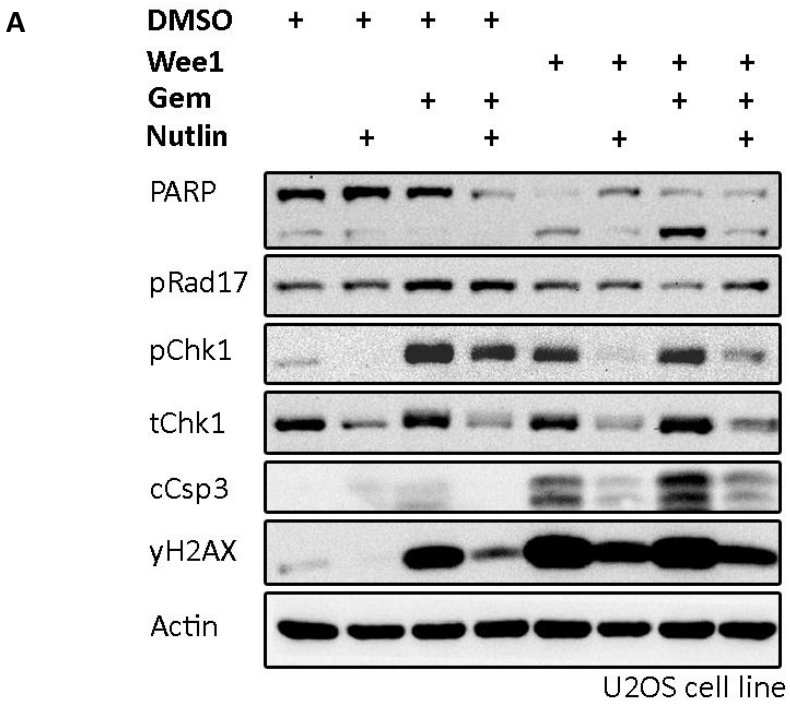


Figure 5.2: Corresponding figure to Figure 5.1.

5.3 Densitometry of the yH2AX signal from Western Blots

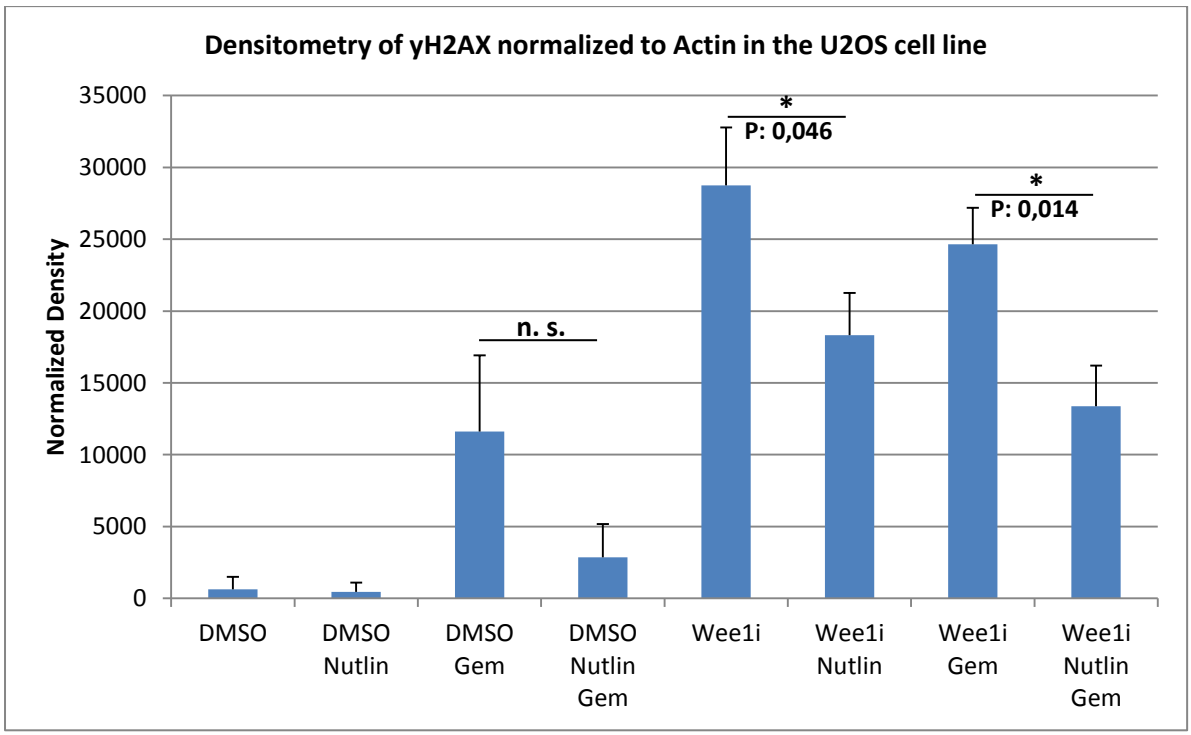


Table 5.1: Nutlin-3a significantly reduces the yH2AX signal upon Wee1i and Wee1i Gemcitabine co-treatment. Error bars represent the standard error (n=3). Significance was assessed by Student's t-test.