THESIS

EFFECTS OF DNA DAMAGE RESPONSE KINASE INHIBITORS ON INCREASED TAXOL HYPERSENSITIVITY LEADING TO CASPASE 3 ACTIVATION

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ABSTRACT

EFFECTS OF DNA DAMAGE RESPONSE KINASE INHIBITORS ON INCREASED TAXOL HYPERSENSITIVITY LEADING TO CASPASE 3 ACTIVATION

Taxol is an antitumor agent that arrests cells in the late G2/M phases of the cell cycle. Our previous research demonstrated that PARP inhibition enhances Taxol induced cell death via oxidative stress and free radical production. In this study, we hypothesized that inhibiting DNA damage response (DDR) kinases would further increase Taxol cytotoxicity by impairing the repair of Taxol-induced DNA damage. We found that inhibition of PI3K-like DDR kinases enhanced Taxol-induced apoptosis through caspase-3 activation. We used Chinese hamster V79 cells and their ATM, ATR, and Ku80-deficient mutants which exhibited hypersensitivity to Taxol. Pharmacological inhibitors, KU55933 (ATM), NU7441 (DNA-PK), and VE821 (ATR), also sensitized V79, CHO, and U2OS human cancer cells to Taxol. This sensitization was associated with increased apoptosis, confirmed by sub-G1 analysis and caspase-3/7 activity assays. Interestingly, MCF7 cells, which lack Caspase-3, did not show enhanced sensitivity to Taxol under DDR inhibition, nor did GM5400 human fibroblast cells. In contrast, MCF7-C3 cells, with restored caspase-3 expression, exhibited significant apoptosis and sensitization, confirming a caspase-3 dependent mechanism. These findings suggest that ATM, ATR, and DNA-PK not only facilitate DNA repair but also suppress Taxol-induced apoptosis via caspase-3. Their inhibition may represent a promising strategy to boost their efficacy of Taxol and potentially enhance responses to radiation therapy through combined targeting of mitotic stress and DDR pathways.

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CHAPTER 1: INTRODUCTION

1.1 Taxol

Paclitaxel, more commonly known as Taxol, comes from the bark of the Western Pacific Yew tree. It was first discovered in 1962 by the National Cancer Institute, who was looking into plant extracts as a natural anticancer treatment. The bark of the tree had the most cytotoxic properties, now known as Taxol, and had the most cytotoxic effects on cancer cells. The natural chemical; however, did not show good results in preclinical trials due to there not being enough extract removed from the bark of the tree. The removal of said chemical was also costly and killed the tree when it was stripped of bark [1]. Another issue seen with natural Taxol in preclinical trials was due to Taxol being insoluble in water. In order to dissolve the extract, castor oil was used which caused anaphylactic reactions. Despite these setbacks, it was concluded that Taxol did help with cancer treatment in 30% of patients causing a high demand for Taxol and leading to the need to create a synthetic Taxol product. A synthetic option of Taxol was then created to maximize its cytotoxicity effectiveness [2].

The creation of synthetic Taxol proved tricky due to Taxol having a strained ring system causing flexibility of the ring. In order to create a strong and viable synthetic Taxol, closure of the ring was needed. The synthesis process performed a series of functional group transformations to stabilize the ring core. Once synthetic Taxol was tested and proved to be useful and cytotoxic to cancer cells, more experimentation was able to be performed in a quick and cost-effective manner [3]. Currently, Taxol has been used to treat lung, ovarian and breast cancers by inducing mitotic arrest through microtubule stabilization causing G2/M cell cycle arrest and leading to apoptosis. It is often combined with Cisplatin treatment and is a promising candidate for cancer co-therapy [2]. Though Taxol has been showing great promise in cancer

treatment, there are still negative side effects, and its mechanism of action (MOA) is still widely unknown which this paper seeks to address.

1.1.2 Structure of Taxol

Taxol contains a large number of oxygen molecules and several ring structures. It has four fused rings at its core made up of a cyclohexene ring, cyclooctane ring, cyclohexane ring, and an oxetane ring [4,5]. Understanding the structure of Taxol is important as it is its structure that enables it to bind to microtubules thus stabilizing said microtubules and preventing cell proliferation. Taxol's N-benzoyl-phenylisoserine side chain is what interacts with the tubulin and is what enabled researchers to develop a synthetic Taxol that utilizes this binding to increase cytotoxicity in cancer cells [6].

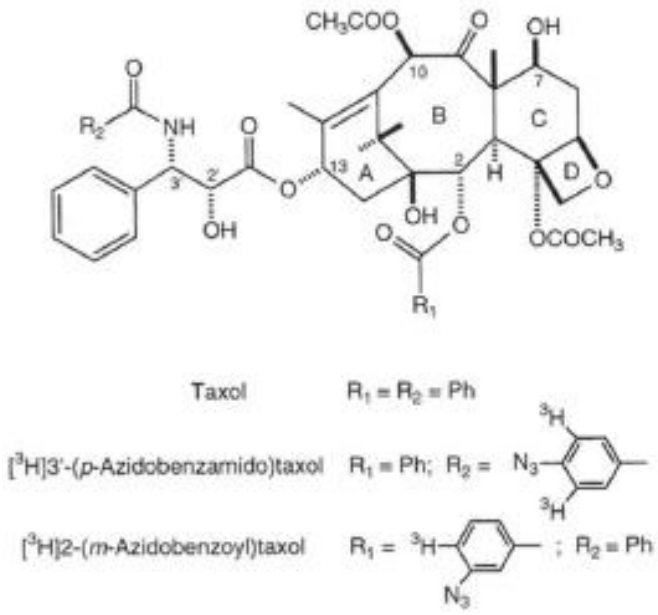

FIG. 1. **Molecular structures of taxol, [^{3}H]2-(*m*-azidobenzoyl)-taxol, and [^{3}H]3′-(*p*-azidobenzamido)taxol.**

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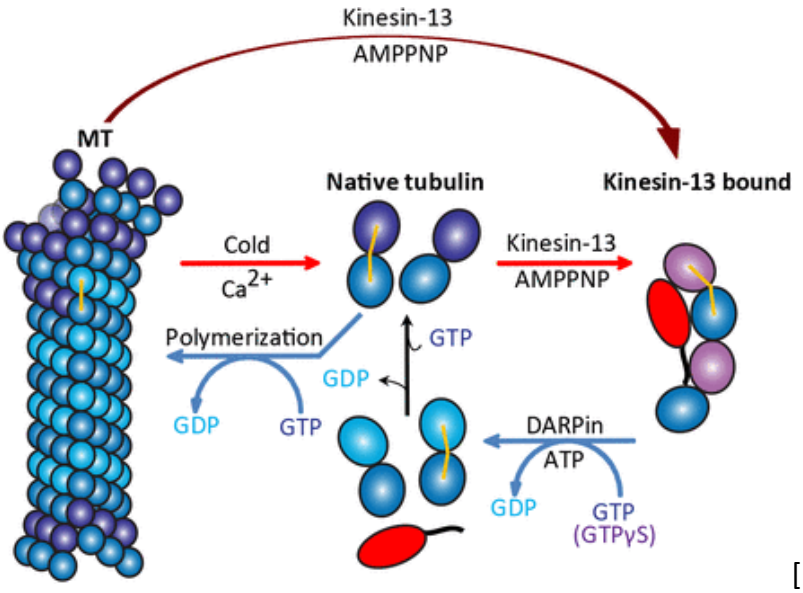
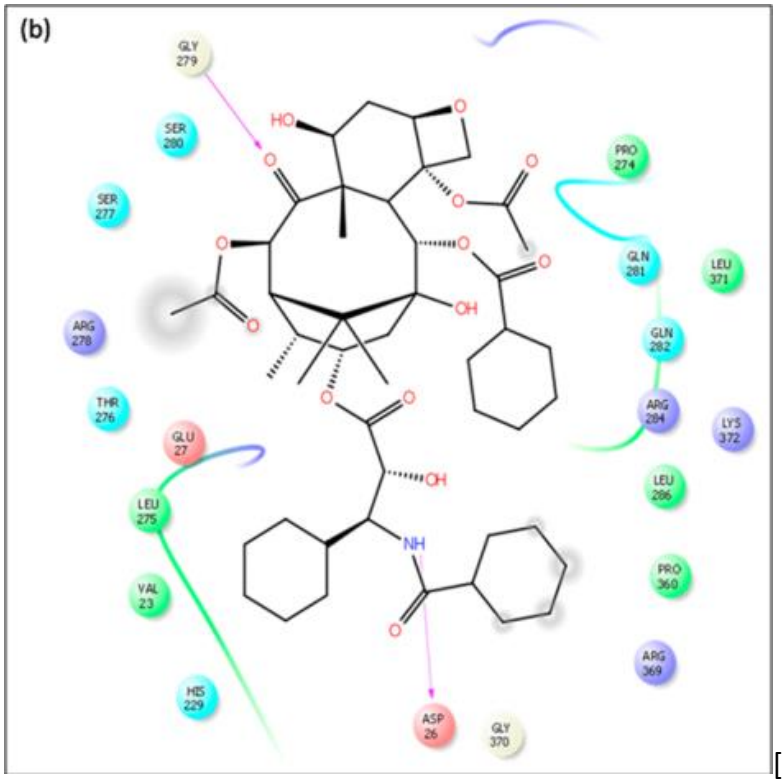
1.1.3 Taxol binding to microtubules

Taxol binds to tubulin in microtubules causing stabilization and preventing separation that occurs during cell division and the stalling of cells in the late G2/M checkpoint causing apoptosis. Tubulin in cells polymerize into microtubules during mitosis and maintain cell shape and the organization of macromolecules and organelles. The process of microtubule function is driven by GTP binding and hydrolysis at the E site of β-tubulin [7]. Taxol binds to the β-subunit in microtubules causing the halting of function by becoming stable and prevents depolymerization by calcium. At very high concentrations of Taxol, bundling of microtubules occurs for cells in Interphase (G1, S, or G2) [7]. Past research saw optimal Taxol stabilization of microtubules at 10 uM concentrations [8]. However, single isotype α1β4 GDP-tubulin lattices are not stabilized but rather expanded. The research helped show that at high concentrations microtubules expand, and an optimal concentration range of Taxol needs to be considered in future research [8]. Any conditions which depolymerize microtubules can inhibit the binding of Taxol suggesting that Taxol specifically binds to cellular microtubules [9].



[10]

Carbons 2, 3, and 7 were seen to be the locations of binding to microtubules [4,5]. The image shows exactly where on β-tubulin Taxol binds. Taxol hydrogen bonds with Gly 279, Asp 26, Asn 204, Gln 11, and Asp 177 with hydrogen binding to Tyr 208 and Tyr 222 in the hydrophobic region [11].



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1.2 Cell cycle

Due to past research identifying cell cycle checkpoint arrest with Taxol, it is imperative to know why this occurs. The cell cycle refers to the process in which cells divide; also known as cell proliferation. The cell cycle has many different parts to it, such as interphase where the cell grows, fixes damages, and gets ready for replication and the mitotic (M) phase where the cell divides. Interphase is split into three sections: G1 (where cell grows), S (where DNA replication and repair occurs), and G2 (where mitotic spindles are organized). Before the cell goes into the

next phase, it stops at what is known as cell cycle checkpoints. These checkpoints halt the cell to make sure it is ready for the next stage and to flag any damage to be repaired or if apoptosis, programmed cell death, needs to be initiated. It has been seen that Taxol arrests cells between the late G2 and M phases. This is due to Taxol causing stabilization of the microtubules, preventing the cell from being able to properly divide and advance to the next stage of the cell cycle leading to apoptosis.

1.2.1 Apoptosis

Apoptosis is programmed cell death. Once activated, the cell begins to shrink and form membranes around sections known as apoptotic bodies or blebs. These blebs begin to break off and are later consumed by white blood cells. This prevents toxins from reaching to other cells and tissues, does not induce inflammation, and is considered a safer method of cell death [12]. Therefore, in cancer research, inducing apoptosis has been a main method for causing cancer cell death while maintaining healthy cells. In cancer cells, apoptosis is often turned off due to cancer cells causing dysfunction of tumor suppressor genes causing uncontrolled growth and evasion of apoptosis. This loss of apoptosis allows cancer cells to survive longer and accumulate more mutations leading to metastasis. There are many ways for cancer to evade apoptosis including inhibiting caspases and overexpression of growth stimulates [13, 14]. This is why treating cancer looks at optimizing apoptosis to the cells leading to tumor death.

1.2.2 Oxidative stress and free radicals

Reactive oxygen species (ROS), otherwise known as free radicals can cause damage to many parts of the cell including lipids, nucleic acids, and proteins causing changes in gene expression and cell proliferation leading to tumor progression [15, 16]. When there is a higher

number of ROS compared to antioxidants in the cell, this can lead to oxidative stress and further damage to the cell and if often seen in cancer patients. Glutathione (GSH) is a main component on finding ROS in cells and maintains intracellular oxidative balance. Past studies used K562 leukemia cells and saw that GSH was reduced in a dose dependent manner with Taxol. However, cells pretreated with antioxidant N-acetyl-L-cysteine (NAC) and Taxol saw restored GSH by 20%. The addition of the NAC antioxidant with Taxol treatment also saw a decline in ROS levels and cytochrome c release. Another study used antioxidants with Taxol treatment on cancer cells to determine if apoptosis was due to oxidative stress and the formation of free radicals. Overall, the addition of antioxidants reduced apoptosis by 20% and they concluded that Taxol increased oxidative stress and the formation of ROS [2]. This finding was confirmed by another study that saw a decrease in cell survival due to the formation of ROS which was reversed by the use of GSH [17].

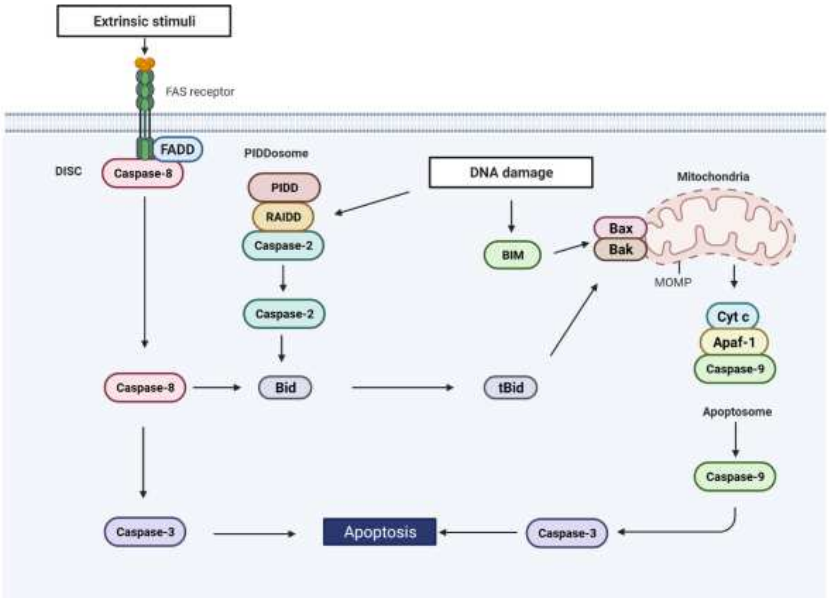
1.3 Extrinsic and Intrinsic pathway

The extrinsic pathway receives the death signal from the plasma membrane and has a ligand bind to its death receptor activating downstream caspases such as caspase 8. Caspase 8, once activated, can then directly cleave effector caspases, like caspase 3/7, and continue towards apoptosis. However, cdk1-mediated phosphorylation of caspase 8 prevents its activation causing the cell to not undergo apoptosis and leads to necrosis, this is seen regularly in colon cancer cells [11]. Research has shown that the extrinsic pathway can also be activated via Natural Killer lymphocytes that are elevated by viral infections. These Natural Killer lymphocytes were seen to be a main indicator cell for the extrinsic pathway and make sure infected cells undergo apoptosis [18]. The intrinsic pathway receives its death signal from the

mitochondria, specifically from the release of cytochrome c. Cytochrome c interacts with an adaptor protein, Apaf-1 which dimerizes and activates caspase 9. Once activated, caspase 9 cleaves and activates downstream effector caspases, such as caspase 3/7 which then leads to apoptosis. Inhibition of caspase 9 leads to nonapoptotic cell death. Past studies have shown that the intrinsic pathway can also detect the presence of viral pathogens which activate the pathway and lead to apoptosis [19].

1.3.1 Caspase 3/7

Caspases 3/7 are activated after an initiator caspase cleaves the effector caspase which can occur either the intrinsic or extrinsic pathway [11]. The effector caspases then act directly on the cell to dismantle the cell. Caspase 3 overlaps with other effector caspases such as 7 which is why they are often mentioned together, though it is unclear exactly how much is shared. The main difference between caspase 3 and 7 that has been found is that amino-terminal sequences in caspase 7 are present in proenzymes, but not in mature enzymes. Past research has shown that either pathway is possible given evidence for oxidative stress which lies in the intrinsic pathway and activation of caspase 9 [12]. It is the intrinsic pathway that is activated via stress and the extrinsic pathway that is activated by ligands [13]. Therefore, understanding both pathways and how caspase 3 is activated is important.


Extrinsic stimuli
FAS receptor
FADD
DISC
Caspase-8
PIDDosome
PIDD
RAIDD
Caspase-2
Caspase-2
DNA damage
BIM
Bax
Bak
Mitochondria
MOMP
Cyt c
Apaf-1
Caspase-9
Apoptosome
Caspase-9
Caspase-8
Bid
tBid
Caspase-3
Apoptosis
Caspase-3

(11)

Chapter 2: Literature Review

2.1 PI3K/AKT/mTOR pathway

The Phosphoinositide 3-kinase/Protein Kinase B (Akt) (PI3K/AKT) pathway regulates cell survival and apoptosis [20]. PI3K is a family of enzymes that regulate cell proliferation and survival and also play a role in signaling Taxol sensitivity [21]. This pathway is overactive in cancer cells and can lead to metastasis. It often cross-talks with other signaling pathways including DDR kinase for DNA repair. In cancer cells, mutations lead to the loss of tumor suppressor genes which alter the pathway leading to continued activation of the PI3K pathway for continued cell growth. PI3K activates AKT to mediate cell growth, AKT then activates mTOR so that more growth factors can be produced as well as increase metabolism in the cell [22]. AKT specifically mediates cell growth and survival by phosphorylating proteins and later activates mTOR. mTOR senses nutrient levels after activation from growth factors and mediates catabolic and anabolic processes maintaining metabolism in the cell. By inhibiting specific kinases in the PI3K pathway such as PARP and DDR kinases, other cell survival pathways would become inhibited leading to decreased tumor cell growth.

2.1.2 PARP inhibition

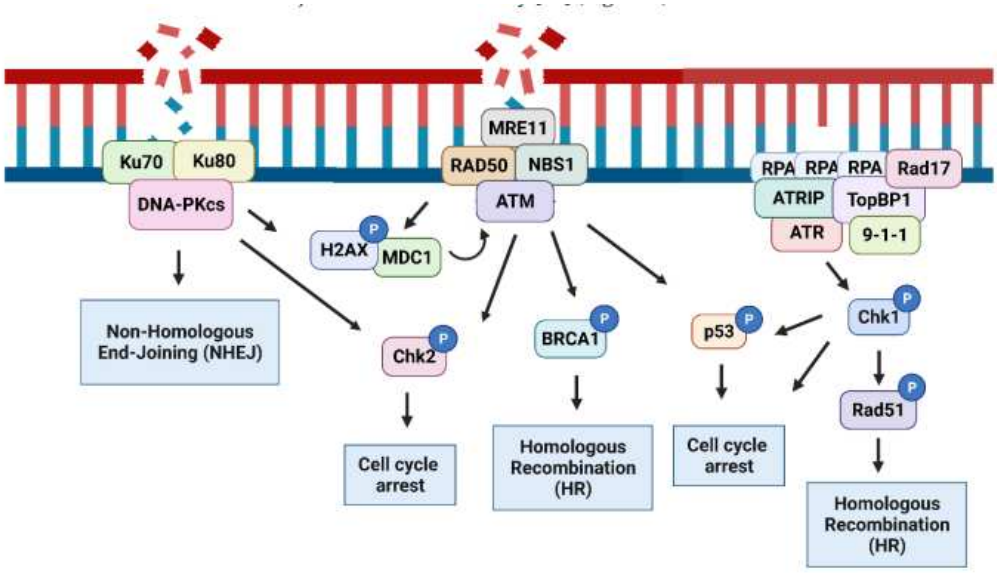
Past research looked at PARP inhibitors which cause homologous repair (HR) deficiencies preventing DNA repair. PARP in cells inhibits single strand break repair (SSBR) and inhibition of PARP was used to clarify the synthetic lethality mechanism and expand PARPs clinical uses as a combination therapy for cancer treatment. DNA damage was confirmed using fluorescence immunocytochemistry of ɣ-H2AX and Rad51 which looked for HR markers [23, 24]. This was confirmed using XRCC8 mutant cells which showed hypersensitivity to PARP

inhibition and Olaparib co-therapy which was similarly seen in BRCA2 mutants [24]. Research also looked at pHH3 of PARP inhibition to show that PARP inhibitors induced DNA damage in the S phase. EdU staining confirmed this, though HR mutant cells did not see a drastic decline in cell survival compared to inhibition of DNA repair systems themselves. Based on this data, PARP inhibitors were combined with Taxol to see if PARP deficiency caused hypersensitivity of cells to Taxol. Growth inhibition was seen in a dose dependent manner with Taxol with the most apoptosis occurring in PARP mutant cells. Cell death also increased in a dose dependent manner with PARP inhibitors [25, 26]. However, these findings only looked at single strand breaks and did not give the full picture which is why DNA damage response (DDR) kinase inhibition was studied in this paper.

2.2 DDR Kinases

DDR kinases respond to double strand breaks that occur due to stress on the cell. This includes but is not limited to radiation, chemicals, oxidative stress, and ROS. The three main DDR kinases are Ataxia-Telangiectasia Mutated kinase (ATM), DNA-dependent Protein kinase (DNA-PK), and Ataxia telangiectasia and Rad3 related (ATR). The damage response signal is phosphorylated by ATM through y-H2AX variant which allows the MDC1 mediator to recruit DDR factors to help fix any damage. However, this is just one of many ways DDR kinases can be activated. Another way is that telomeres recruit DDR kinases in the G2 phase of the cell cycle to reconstruct the end protection complex and fix any DNA damage [27]. Due to the many areas where DDR can be found, it is important to look at its interactions with caspase 3/7 and the apoptosis pathway. DDR kinases are located on membranes where signals that are produced from DNA damage activate said kinases. These kinases then activate repairs as well as downstream

signals for cell cycle arrest and further repairs [28]. The connection between DDR kinase and Taxol leading to apoptosis is what this paper seeks to address.



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2.2.1 ATM inhibitor (KU 55933)

ATM is the first line of defense for DDR kinases. It often recruits other DDR kinases like DNA-PK and ATR after a DSB. Once DNA damage occurs, ATM signals to halt the cell in either the G1, S, or G2 cell cycle checkpoint and then activates DNA repair mechanisms [26]. ATM is activated by the MRN protein complex when DSBs occur [27]. Once activated, it phosphorylates and activates downstream effector proteins such as p53, Chk2, Brca2, and H2AX causing cell cycle checkpoint arrest and eventual DNA repair. Cancer cells have a large upregulation of Akt which is a large component in the phosphoinositide 3-kinase (PI3K) pathway making it a good target for cancer treatment [28]. KU 55933 is a specific ATM inhibitor that shows selectivity for ATM that is stronger than other similar kinase inhibitors. The inhibitor arrests the cell in the G1 phase and downregulates cyclin D1 leading to apoptosis. Though it downregulates cyclin D1, it does not degrade or decrease the transcription of cyclin D1. However, past research also saw

inhibition of Akt and a reduction in cell proliferation in cancer cells which was believed to be due to this downregulation of cyclin D1 [28]. Past research saw that KU55933 on its own did not show increased cytotoxicity [29]. Based on its inhibition of Akt, KU55933 is considered a good DDR kinase inhibitor to use to target ATM kinases in the DDR kinase pathway with Taxol co-treatment.

2.2.2 DNA-PK inhibitor (NU7441)

DNA-dependent protein kinase (DNA-PK) are DDR kinases that repair DSBs. DNA-PK repairs DSBs through non-homologous end joining (NHEJ) which occurs throughout the cell cycle. NHEJ repairs DNA by directly joining broken strands of DNA without the need of a template, because there is not a template being used, it is prone to making errors. It is made up of two heterodimers Ku70 and Ku80 which activate DNA-PK catalytic subunits which create structural platforms for repair reactions through the binding and bridging of two DNA strands at the damaged area [27]. Pre-clinical trials have shown that DNA-PK inhibition has increased chemo and radio-sensitization to thoracic, head, or neck tumors [30]. Inhibition of DNA-PK is thought to be a good approach to radiation resistant cancer cells. NU7441 is an inhibitor of DNA-PK which prevents autophosphorylation of DNA-PK preventing DNA DSB repair. Past studies have shown decreased DNA repair with NU7441 in a dose dependent manner. The inhibition also prevented the recruiting of other DNA repair systems that DNA-PK activates downstream [31].

2.2.3 ATR inhibitor (VE821)

ATR is a DDR kinase inhibitor that has shown to decrease malignancies. Inhibition of ATR increases the development of DNA damage in cells in the S phase of the cell cycle and

prevents repair. Cells that are ATR deficient often see S phase arrest that leads to fork stalling and replication failure [32]. It is often activated at stalled DNA forks where it stabilizes and repairs the stalled forks [33]. VE-821 is an ATR inhibitor that directly inhibits the ATR-CHK1 pathway with high selectivity and specificity. This inhibitor prevents S phase activation and cells stopping in the G2/M checkpoint, allowing cells to continue onto mitosis with DNA damage and leading to cell death. Past studies have shown increased cytotoxicity in various cancer cells and decreased cell viability in a dose dependent manner over time [33].

2.3 Cell lines

2.3.1 V79 Cells

Chinese hamster V79 lung cells were used as a control in the experiments as well as their ATM (M1-10), ATR (R1-34), and Ku80-deficient mutants (XR-V15B). M1-10 is an ATM knockout, R1-34 is ATR deficient, and XR-V15B is a Ku80 mutant. V79 cells are commonly used in experimentation due to their ease of use and because they are good indicator cells to determine mutations, chromosomal aberration, and cytotoxicity. V79 cells lack cytochrome P450 which prevents them from naturally metabolizing and detoxifying drugs [34]. This makes them excellent candidates for understanding how Taxol affects cells that lack cytochrome P450 such as certain tumor cells, lungs, and skeletal muscle. Past research using Taxol with V79 cells did show a dose dependent decline and a decrease in colony formation ability. They also saw an increase in LDH activity confirming the formation of micronuclei after Taxol treatment [35]. Other research looked at V79 with Cisplatin treatment, a commonly used cancer treatment drug often used alongside Taxol. They saw that V79 cells had a lower sensitivity to death by Cisplatin which was believed to be due to inhibition of caspase dependent pathways [36]. This resistance further shows the need for better cancer treatment and co-therapy with Taxol.

2.3.2 M1-10 cells

M1-10 cells are an ATM knockout (ATM -/-) cell meaning that they do not contain any ATM kinases. ATM kinases respond to DNA damage signaling mainly double stand breaks (DSBs). It is activated by trans-autophosphorylation that causes a switch from an inactive protein complex to an active kinase causing cell cycle arrest, DNA repair, and even apoptosis. ATM is able to sense ROS accumulation and deficiency in ATM causes increased generation of mitochondrial ROS [37]. Past research saw that animals with a higher cytotoxic T cell function saw increased survival despite ATM -/-. The research showed that targeting ATM -/- agents were able to increase survival of cells and the role ATM plays in cell survival [38]. With past research showing Taxol induced oxidative stress and the formation of ROS, observing Taxol effects on an ATM -/- cell line would help determine increased cytotoxicity due to oxidative stress and ROS. Furthermore, preclinical studies showed that ATM inhibition alone did not see cancer cytotoxicity; however, co-treatment could show great potential [29].

2.3.3 R1-34 Cells

R1-34 cells are ATR deficient cells meaning the cells have ATR but, it is insufficient and is unable to properly perform its function as a DNA repair kinase. ATR is activated from many types of DNA damage including DSBs, base adducts, crosslinks, and replication stress. ATR signaling depends on co-localization of the ATR-ATRIP complex that recognizes a DNA end and the DNA damage. The primary role of ATR is checkpoint activation and localization of ATR and ATM and is the key to the regulation of said kinases [39]. Therefore, utilizing ATR deficient cells with Taxol will help clarify the MOA of Taxol cytotoxicity in cells compared to cells with intact ATR kinases. Past research saw that inhibition of ATR caused replication stress, DNA damage, and genomic instability in ARID1A gene deficient cells [40]. Another paper looked at caffeine to

inhibit ATR and increase cell cytotoxicity. They saw that caffeine was able to inhibit ATR and showed the route of ATRs therapeutic target in cancer cells through premature chromatin condensation [41].

2.3.4 XR-V15B Cells

XR-V15 cells are a Ku80 mutant cell. Ku80 repairs DNA ends through NHEJ by binding to DNA ends, nicks, and gaps. It forms a DNA-PK complex and when deleted, is very sensitive to radiation and is unable to repair DSBs. Past research on Ku80 defective mice showed premature aging, hypersensitivity to radiation, and an increase in ROS production [42]. Past research saw that KU80 mutants still maintained their KU70 counterparts; however, KU70 alone was not able to accumulate DSBs and required KU80 to detect DSBs and leads to DNA repair [43]. Another research looked at KU80 mutant zebra fish and saw that alone, the KU80 mutant did not lead to apoptosis, but in combination with radiation treatment, apoptosis occurred [44]. Research on Taxol cytotoxicity for this variant helps to better understand the MOA of Taxol and if the use of DDR kinase inhibitors may increase Taxol cytotoxicity.

2.3.5 CHO cells

Chinese hamster ovary (CHO) cells were first established in 1956 with many variants being made since. Work with CHO cells dramatically increased with the development of recombinant DNA technologies in the 1980s. They have a low chromosome number making them easy to work with and good for genetic testing. CHO cells have a high capacity for efficient post-translational modification and produce recombinant proteins that are compatible and bioactive in humans making them good cells for studying cytotoxic effects for pharmaceuticals. They are commonly used to develop therapeutics and have been used to test various chemicals

for leukemia, hormone imbalance, and other cancers. CHO cells are able to be engineered to target specific antibodies which makes them great for target therapy research [45]. For example, CHO cells have been engineered to influence the mTOR complex 1 [46]. The main method of CHO engineering comes from CRISPR technology which focuses on glycosylation modulation, productivity enhancement, and developing antibiotic free selection systems. This has enabled many different CHO therapy targets and advances in genome editing [47]. This is why CHO cells are an important cell to observe cytotoxicity effects of Taxol.

2.3.6 MCF-7 and MCF7-C3 cells

MCF-7 cells are human epithelial breast cancer origin cells which lack a caspase 3 and were used alongside MCF7-C3, which has restored caspase 3, to determine if Taxol cytotoxicity occurs via the caspase 3 pathway. Past studies have shown that MCF-7 cells are resistant to Cisplatin, a common cancer treatment drug [48]. They are good for determining if a chemical activates the caspase 3/7 pathway and are often used alongside their caspase 3 restored cell counterpart, MCF7-C3. MCF-7 cells were first discovered in 1973 at the Michigan Cancer Foundation when they were isolated from a woman with metastatic disease. It is an ER-positive and progesterone receptor with large variations in chromosome numbers [49].

MCF7-C3 cells have had caspase 3 reconstructed and is often used to resensitize MCF-7 cells to treatments. They help to show that caspase 3 activation is crucial for induced apoptosis after cancer treatments including Taxol [50]. Past research has shown that the lack of caspase 3 in MCF-7 cells may be what causes them to be chemotherapeutic resistant. This resistance is a major issue in cancer treatment and by restoring the caspase pathway, this resistance can be overcome. This paper uses MCF-7 and MCF7-C3 to determine if Taxol cytotoxicity is due to the activation of the caspase 3 pathway.

2.4 Preclinical trials with Taxol

Current cancer treatments use Taxol in combination with other therapies such as Cisplatin when treating breast and ovarian cancers. However, Taxol has been seen to cause various side effects including neuropathy, paresthesia, and pain. Neuropathy was also seen to be higher in diabetic patients and high doses of Taxol was considered neurotoxic. This may be due to the high dose of the Taxol with Cisplatin of around 135-175 mg/m^2 [51]. Other side effects seen include myelosuppression due to Taxol lowering white blood cell counts at 135 mg/m^2. Though this effect on the bone did not lead to sepsis or death it is worth considering when developing cancer treatment [52] Taxol has also been used in treatment due to certain cancers, such as MDR protein containing breast cancers, to be drug resistant. Using Rg3 liposomes with Taxol, in vitro, MCF-7/T tumor cells were able to inhibit cell proliferation. They saw an inhibition of angiogenesis and showed that combination therapy can cause cytotoxicity in drug resistant cancer [53]. The addition of DDR Kinase inhibitors could help lower clinical doses of Taxol and provide better co-treatment.

2.5 Hypothesis

Based on past research, we hypothesize that inhibiting DDR kinases could enhance Taxol-induced cytotoxicity through the disruption of DNA repair pathways and/or activation of apoptosis that was seen with other PI3K family members. In order to test this, isogenic Chinese hamster V79 mutant cell lines that were deficient in ATM, ATR, or Ku80, V79 cells, CHO cells, U2OS, MCF7, MCF7-C3, GM5400, and ATM, ATR, and DNA-PK inhibitors were used in combination with Taxol. Our research showed that inhibition of ATM, ATR, and DNA-PK increased sensitivity of cells to Taxol induced apoptosis in a caspase 3 dependent manner.

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Chapter 3: Manuscript

3.1 Introduction

As mentioned in the introduction, Taxol comes from the bark of the Western Pacific Yew tree through a plant extract screening program [1]. Unlike other major anti-tumor agents that exert their effects by inducing lethal DNA damage, Taxol's primary mechanism of action is distinct in that it stabilizes microtubules by inhibiting tubulin depolymerization, leading to cell cycle arrest in the late G2 and M phases [2]. Because of its unique mechanism involving mitotic arrest, Taxol is often combined with other DNA-damaging anti-tumor agents. For example, combinations with gemcitabine and radiation have been employed in pancreatic and breast cancer therapy [5,6]. A more common co-therapy is with Cisplatin, a common chemotherapeutic drug. However, Taxol and Cisplatin treatment have seen the development of neuropathy at doses higher than 175 mg/m^2 Taxol [27]. Despite these effects, combination therapies are a promising treatment option for tumors that have developed resistance to individual treatments.

Past research showed that PARP inhibitors enhanced Taxol-induced cytotoxicity when used in combination [3]. Additionally, inhibition of Akt has been reported to enhance Taxol-induced apoptosis in ovarian cancer cells [7]. Increased Taxol efficacy was also observed following JNK inhibition, which activated caspase-9 and caspase-3, leading to apoptosis [8]. These findings suggest that activating apoptotic pathways, particularly those involving the PI3K/AKT/mTOR signaling axis, may be an effective strategy to boost Taxol sensitivity.

While the role of canonical PI3K signaling in Taxol sensitivity has been studied [9], the potential contribution of PI3Krelated kinases, especially those involved in the DNA damage responses (DDR) remains unclear. In particular, DDR-associated PI3K-like kinases such as

ATM, ATR, and DNA-PK, play essential roles in DNA repair, checkpoint control, and cell fate decisions following DNA damage as mentioned above [10]. Inhibition of these DDR kinases block DNA repair and disrupt checkpoint signaling [11–13]. Due to this disruption, we hypothesized that inhibiting DDR kinases would enhance Taxol-induced cytotoxicity through disruption of DNA repair pathways and/or activation of apoptosis, similar to other PI3K family members. To test this, we used isogenic Chinese hamster V79 mutant cell lines deficient in ATM, ATR, or Ku80, along with selective inhibitors of ATM, ATR, and DNA-PK in combination with Taxol. Our study revealed that inhibition of ATM, ATR, or DNA-PK sensitize cells to Taxol-induced apoptosis in a caspase-3 dependent manner.

3.2. Materials and methods

3.2.1 Cell culture

Chinese hamster ovary origin CHO10B2 and Chinese hamster lung origin V79 cells were kindly supplied by Dr. Joel Bedford of Colorado State University (Fort Collins, CO). V79 mutant M1-10 and R1-34 cells were isolated previously [14]. XR-V15B cells were purchased from Coriell Institute for Medical Research (GM16144) [15]. M1-10 is an ATM knockout, R1-34 is ATR deficient, and XR-V15B is a Ku80 mutant. Human osteosarcoma origin U2OS and human breast cancer origin MCF7, caspase-3 restored MCF7-C3, and GM5400 (normal human fibroblast cells) [16] were kindly supplied by Dr Joel Bedford. Naringin and AA2G were kindly supplied by the Toyo Sugar Refining company in Tokyo Japan. Cells were maintained in Alpha MEM (Hyclone, Thermo Fisher Scientific, Waltham, MA) with 10 % heat inactivated Fetal Bovine Serum (Sigma-Aldrich, St Louis, MO), antibiotics (Anti-Anti; Invitrogen, Waltham, MA), and were cultured at 37 °C with 5 % CO2 and humidity.

3.2.2 Chemicals

Taxol was purchased from Nippon Kayaku (Tokyo, Japan). The ATM inhibitor KU55933 [13], ATR inhibitor VE-821 [17], and DNA-PK inhibitor NU7441 [11] were obtained from Selleck Chemicals (Houston, TX). Those inhibitors were used at their respective maximum tolerated concentrations for each cell line. Those chemicals were dissolved in DMSO. The caspase-3 inhibitor Ac-DEVD-CHO [18] was purchased from TargetMol Chemicals (Wellesley Hills, MA) and used at a concentration of 100 μM to inhibit caspase-3 activity, described in previous studies [19,20].

3.2.3 Growth inhibition

To determine the doubling time and effect of growth inhibition, 30,000 cells were plated into each well of a 12-well plate with 0, 10, or 20 nM of Taxol. Cells were incubated at 37 °C. Cell number was obtained through the Coulter Counter Z1 (Beckman Coulter, Indianapolis, IN) every 24 hrs for 4 consecutive days. Cell doubling time was calculated using GraphPad Prism 8 with an exponential growth equation (GraphPad Software, San Diego, CA).

3.2.4 Clonogenic cell survival

Single cells were plated to generate 50–300 colonies. Various concentrations of chemicals were added to the cell culture media. Cotreatment was carried out by adding two agents at the same time. Then Chinese hamster cells were cultured for 1 week and human origin cells were cultured for 10 days for colony formation. The colonies were fixed and stained using 100 % ethanol followed by 0.1 % crystal violet. Macroscopic colonies containing more than 50 cells were considered survivors as previously described [21]. Regression curves were drawn from cell survival fraction using GraphPad Prism 8 software.

3.2.5 Apoptosis measurement

Apoptosis induction by Taxol was assessed by measuring caspase activation and analyzing the sub-G1 population [22]. Log-phase growing cells were treated with 10 nM Taxol. After 48 h of incubation, caspase 3/7, 8, and 9 kits (Promega, Madison, WI) were used to detect the activation of caspase 3/7, 8, and 9, to determine the mechanisms of early apoptosis. Luminescence from 10000 cells were measured using a Lumat LB9507 (Berthold technologies, Oak Ridge, TN). After 48 hrs of drug treatment, cells were trypsinized and fixed in 70 % ethanol in PBS and stored at −20 °C until analysis. Cells were centrifuged at 1500 rpm for 5 min and washed with PBS. Cells were then stained with 0.5 mL of 20 μg/mL propidium iodide and 0.5 mg/mL RNase A for 30 min before analysis. 30000 cells were analyzed by Cyan ADP cytometer (Beckman Coulter). Sub-G1 population was obtained in Summit software (Beckman Coulter).

3.2.6 Metaphase arrest analysis

After 24 hrs of drug treatment, cells were trypsinized and fixed in 70 % ethanol in PBS and stored at −20 °C until analysis. Cells were centrifuged at 1500 rpm for 5 min and washed with PBS. Cells were stained with rabbit Anti-Histone H3 (phosphor S10) antibody (ab5176, Abcam, Waltham, MA) and Alexa 594 conjugated anti-rabbit secondary antibody. Cells were then analyzed by Cyan ADP flow cytometer to obtain phosphoHH3 positive population. At least 30000 cells were analyzed by data point in Summit Software.

3.2.7 Cytogenetic analyses

For chromosomal aberration (CA) analysis, exponentially growing cells were treated with Taxol, and metaphase chromosomes were prepared every 3 hrs, three times, following Colcemid arrest. To determine whether metaphase cells originated from S-phase, 10 μM of EdU was added

during the treatment. Metaphase cells collected at the first 3-hr time point are expected to be predominantly derived from cells that were in G2 phase at the time of Taxol treatment, while those collected at the final 3-hr time point are expected to be predominantly derived from cells initially in S phase. Metaphase cells were harvested after 3 hrs of treatment with 0.1 μg/mL Colcemid. The cells were then subjected to hypotonic treatment with 75 mM KCl and fixed using Carnoy's fixative (methanol: acetic acid, 3:1), according to standard cytogenetic protocols [23]. Fixed cells were spread on glass slides and stained with 5 % Giemsa solution. To assess cell cycle delay, the mitotic index and the fraction of EdU positive metaphase cells were analyzed. The mitotic index was calculated from a total of 300 cells, while EdU incorporation was assessed in 50 metaphase cells. Chromosomal aberrations were scored using a Zeiss Axioskop microscope at 100X magnification with at least 50 metaphase spreads analyzed per experiment in three independent experiments.

3.2.8 Statistics

All experiments were carried out at least three times and error bars indicate standard error of the means. Data was analyzed using Prism 6 software for one-way or two-way ANOVA analysis; p-values <0.05 were categorized as being statistically significant.

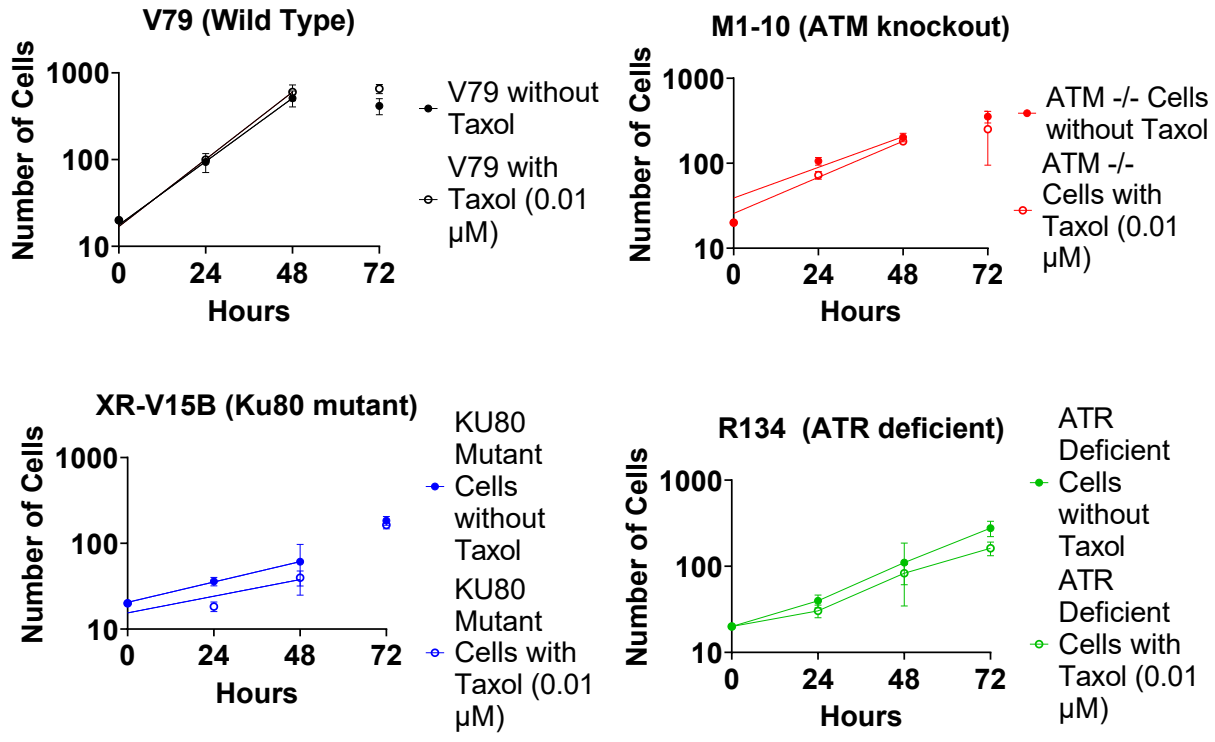
3.3 Results

3.3.1 Enhancement of Taxol induced growth inhibition and cytotoxicity by DDR inhibitors

To assess the impact of DDR kinase deficiency on Taxol sensitivity, we examined cell growth and colony formation in V79 wild-type cells and DDR kinase–deficient mutants (M1-10, R1-34, and XR-V15B). We expected to see a decrease in overall growth in each mutant cell line and with the addition of each DDR inhibitor compared to the V79 wild type cells. All DDR

kinase–deficient mutants exhibited slower proliferation than wildtype cells (Fig. 1A), reflecting the essential role of these kinases in cellular proliferation. Treatment with Taxol further reduced proliferation in all lines, with the greatest effects observed in DNA-PK and ATR deficient cells (Fig. 1A). Pharmacological inhibition of ATM, ATR, or DNA-PK by KU55933, VE821, and NU7441, respectively, reduced proliferation in wild-type V79 cells, and co-treatment with Taxol produced a greater growth-inhibitory effect than either treatment alone (Fig. 1B). Colony formation assays confirmed that both genetic deficiency in V79 cells and CHO cells and pharmacological inhibition of DDR kinases for V79 cells significantly enhanced Taxol-induced cytotoxicity, with DDR inhibitors producing a stronger sensitizing effect than genetic mutations (Fig. 3A).

A



B

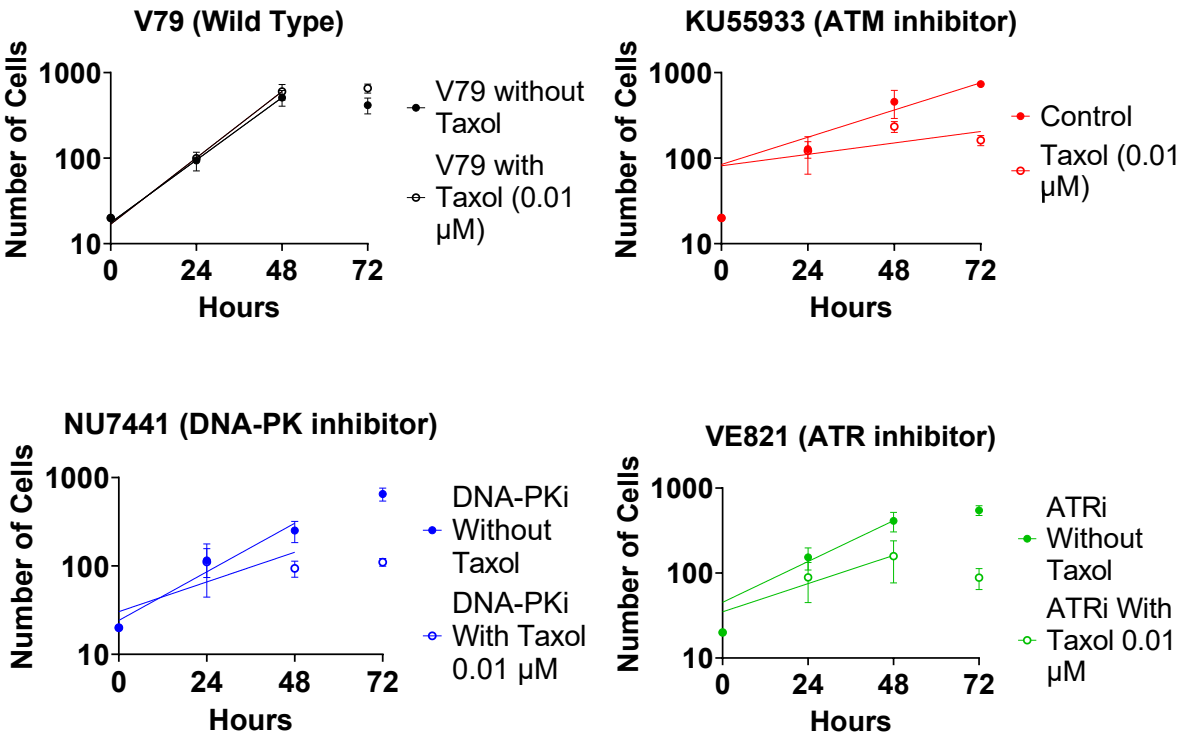


Fig. 1. Enhancement of cytotoxicity to Taxol by DDR kinase deficiency

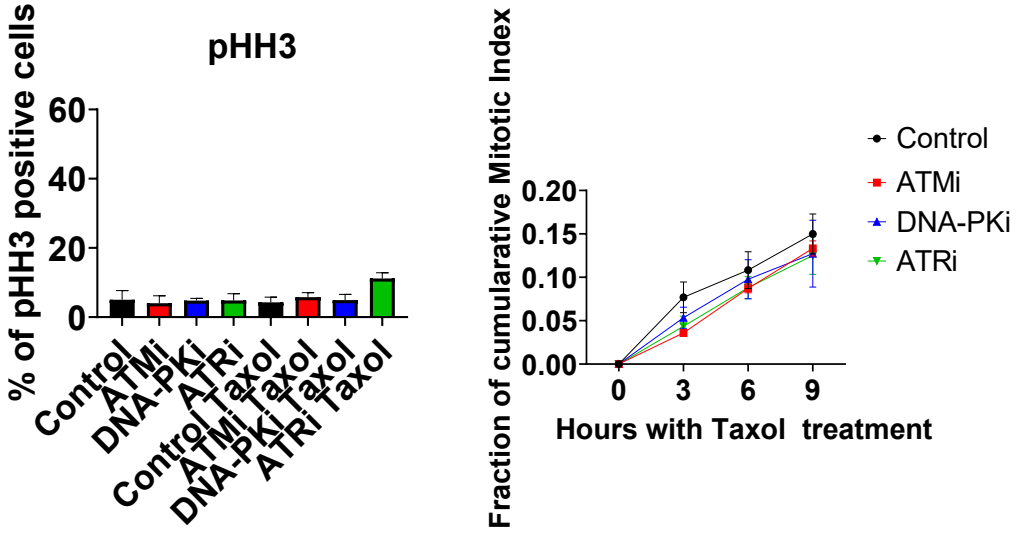
A) Growth inhibitions in V79 and their mutants with 10 nM Taxol treatment. V79 (wild type), M1-10 (ATM mutant), R1-34 (ATR mutant), and XR-V15B (Ku80 mutant) cells for 4 days.

B) Growth inhibitions in V79 cells treated with 10 nM Taxol and DDR kinase inhibitors: KU55933 (5 μM ATM inhibitor), VE821 (5 μM ATR inhibitor), or NU7441 (2.5 μM DNA-PK inhibitor) for 4 days.

3.3.2 Enhancement of Taxol induced cytogenetic damage by DDR inhibitors

Given the known roles of ATM and ATR in checkpoint regulation, we investigated whether DDR inhibition would alter mitotic entry and chromosomal integrity in V79 cells (Fig. 2). It was thought that the DDR kinases prevent Taxol induced cytotoxicity through their checkpoint repair and this study was used to confirm this idea. Analysis of mitotic index and S-phase progression

revealed that DDR inhibition did not significantly accelerate entry into mitosis under our experimental conditions as was previously thought. In contrast, co-treatment with DDR inhibitors and Taxol markedly increased chromosomal aberrations, detectable as early as 3 hrs after treatment (Fig. 2). The timing of peak aberrations differed depending on the specific kinase inhibited, indicating that the enhancement of chromosomal damage by DDR inhibitors is not simply due to checkpoint override. Continuous Taxol treatment alone or combined with DDR inhibitors did not significantly increase the fraction of phosphorylated Histone H3 (PHH3)-positive cells, indicating limited mitotic accumulation in the tested condition (Fig. 2). These observations suggest that DDR kinases protect against Taxol-induced genomic instability through mechanisms independent of major checkpoint functions.



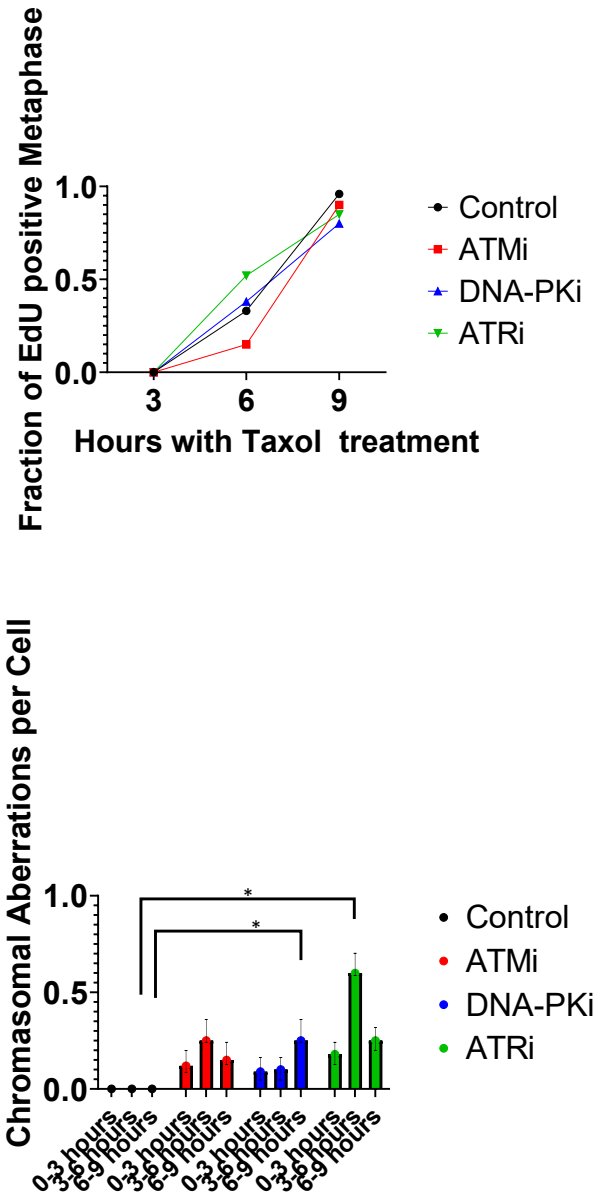


Fig. 2. Cytogenetic analysis of Taxol in combination with DDR kinase inhibitors

Experimental protocol for drug treatments. Cells were treated with 10 nM Taxol and DDR kinase inhibitors: KU55933 (5 μM), VE821 (5 μM), or NU7441 (2.5 μM) Percentage of phosphorylated Histone H3 (PHH3)-positive cells after 24 h, measured by flow cytometer. Mitotic cell

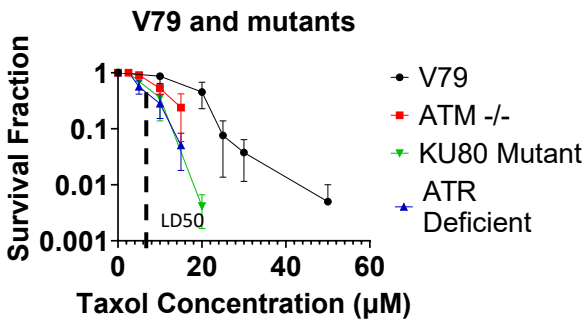
accumulation after treatment with Taxol and DDR kinase inhibitors. Entry to mitosis from S-phase cells after treatment with Taxol and DDR kinase inhibitors. Chromosomal aberrations after Taxol and DDR kinase inhibitor treatments.

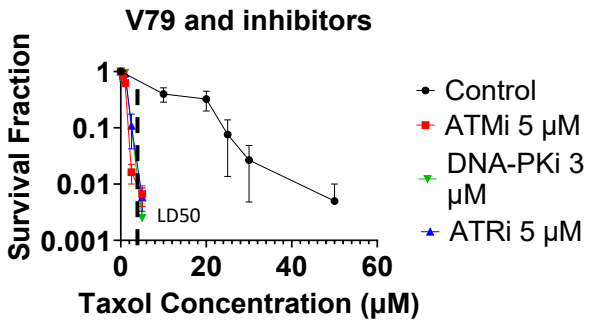
Independent experiments were carried out at least three times. Error bars indicate SEM. * indicates statistically significant differences ($p < 0.05$).

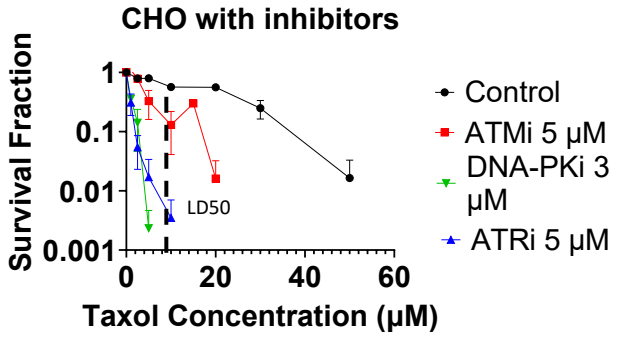
3.3.3 Requirement of caspase-3 for Taxol sensitization by DDR inhibitors in human cancer cells

To determine whether caspase-3 is essential for DDR inhibitor–mediated sensitization, we tested three human cancer cell lines, U2OS, GM5400, and MCF7. We hypothesized that restoration of caspase 3 would increase cytotoxicity and co-therapy treatment would greatly sensitize both the U2OS and GM5400 cell lines. Similar to hamster cells, U2OS cells display enhanced Taxol sensitivity when co-treated with DDR inhibitors (Fig. 3B). In contrast, MCF7 cells, which lack caspase-3, showed no increase in cytotoxicity. Restoration of caspase-3 expression in MCF7 (MCF7-C3) reinstated the sensitizing effect of DDR inhibitors and Taxol co-treatment, as confirmed by protein expression and sub-G1 population analysis (Fig. 3A). Among the inhibitors tested, ATR inhibition with VE821 produced the most pronounced apoptotic response. These findings establish that caspase-3 is required for DDR inhibitor–enhanced Taxol-induced apoptosis in human cancer cells. Normal human fibroblast, GM5400, cells were also studied to determine Taxol and DDR inhibitor effects on non-cancerous cells (Fig 3B). There was little to no increases in cytotoxicity with the DDR inhibitors and Taxol. This shows that normal human fibroblast cells may not be as sensitive to Taxol and DDR inhibition co-therapy making the treatment viable for clinical use.

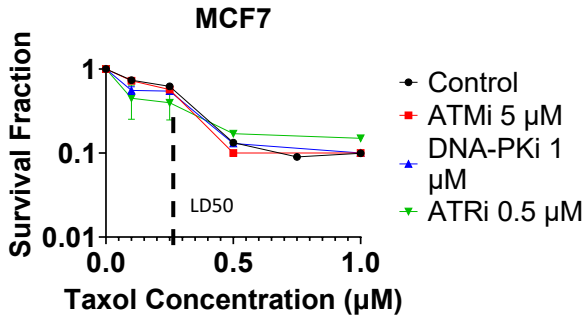
A

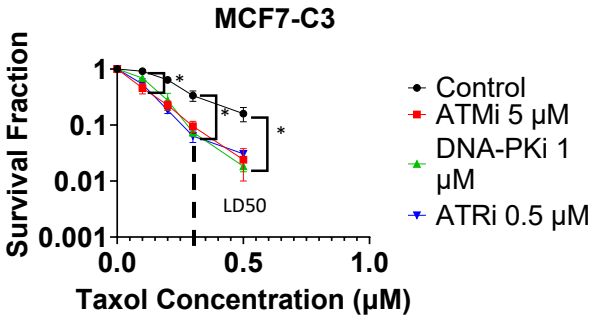

V79 and mutants
Survival Fraction
1
0.1
0.01
0.001
LD50
0
20
40
60
Taxol Concentration (μM)
V79
ATM -/-
KU80 Mutant
ATR Deficient


V79 and inhibitors
Survival Fraction
1
0.1
0.01
0.001
LD50
0
20
40
60
Taxol Concentration (μM)
Control
ATMi 5 μM
DNA-PKi 3 μM
ATRi 5 μM


CHO with inhibitors
Survival Fraction
1
0.1
0.01
0.001
LD50
0
20
40
60
Taxol Concentration (μM)
Control
ATMi 5 μM
DNA-PKi 3 μM
ATRi 5 μM

B


MCF7
Survival Fraction
1
0.1
0.01
LD50
0.0
0.5
1.0
Taxol Concentration (μM)
Control
ATMi 5 μM
DNA-PKi 1 μM
ATRi 0.5 μM


MCF7-C3
Survival Fraction
1
0.1
0.01
0.001
LD50
*
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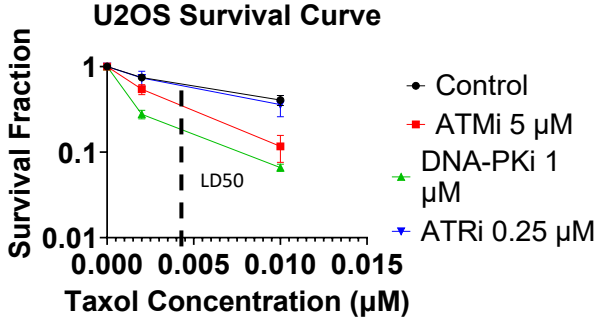
*
0.0
0.5
1.0
Taxol Concentration (μM)
Control
ATMi 5 μM
DNA-PKi 1 μM
ATRi 0.5 μM


U2OS Survival Curve
Survival Fraction
1
0.1
0.01
LD50
0.000
0.005
0.010
0.015
Taxol Concentration (μM)
Control
ATMi 5 μM
DNA-PKi 1 μM
ATRi 0.25 μM

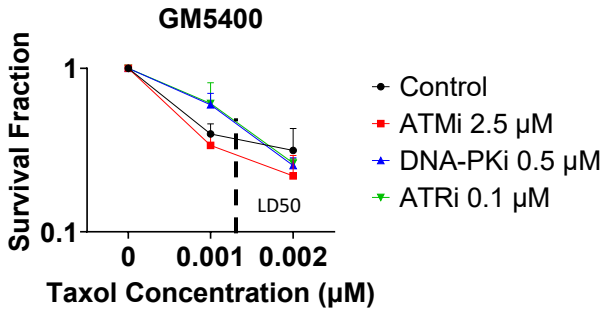

GM5400
Survival Fraction
1
0.1
LD50
0
0.001
0.002
Taxol Concentration (μM)
Control
ATMi 2.5 μM
DNA-PKi 0.5 μM
ATRi 0.1 μM

Fig. 3. Taxol and DDR kinase inhibitor-induced cytotoxicity in human cells

A) Clonogenic cell survival of V79 and CHO cells treated with inhibitors, and V79 mutants. Cells were continuously exposed to Taxol and inhibitors during colony formation for 7 days.

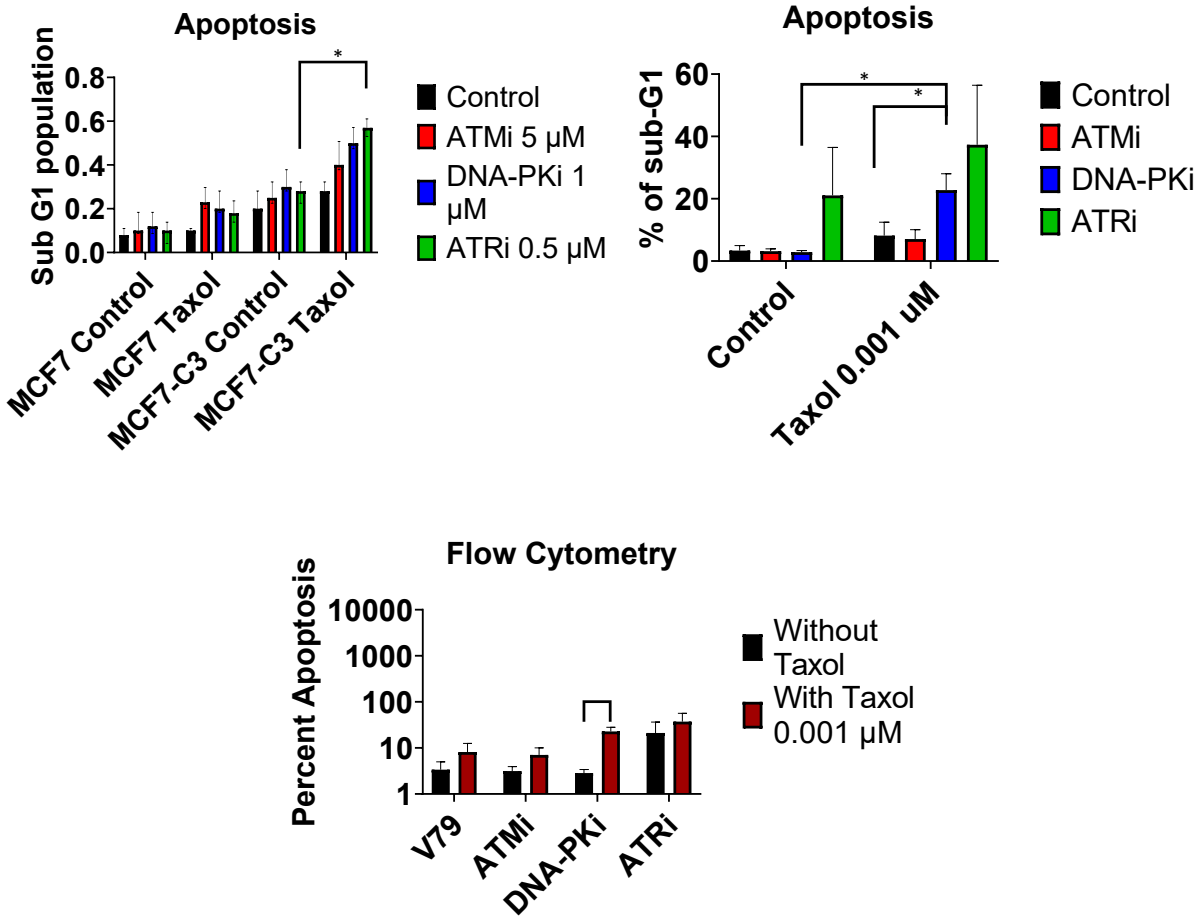
B) Clonogenic cell survival curves of U2OS, GM5400, MCF7, MCF7-C3 cells.

Independent experiments were performed at least three times. Error bars indicate standard error of the mean (SEM).

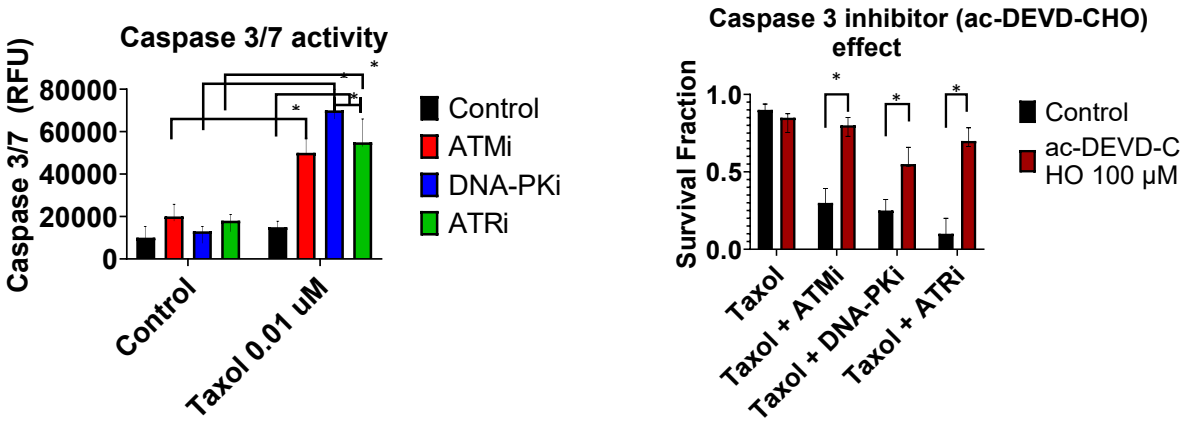
3.3.4 Enhancement of Taxol induced apoptosis through caspase 3/7

To evaluate cell cycle progression and apoptotic responses, we analyzed sub-G1 populations, and caspase activities in V79 cells. We expected to see increased apoptosis with the activation of caspase 3 after Taxol treatment which would be further increased with the addition of DDR inhibitors. We also expected the caspase 3 activation to be inhibited thus increasing survival with ac-DEVD-CHO caspase 3 inhibitor. The activation of caspase 3 was thought to be activated via the intrinsic pathway and activation of caspase 9 due to past research seeing increases in oxidative stress which occurs in the same pathway. Co-treatment with DDR inhibitors significantly elevated the sub-G1 fraction, particularly in cells treated with DNA-PK or ATR inhibitors (Fig. 4A). Measurement of caspase activity demonstrated that caspase-3/7, but not caspase-8 or -9, was strongly activated under co-treatment conditions (Fig. 4B). Pharmacological inhibition of caspase-3/7 abrogated the enhanced cytotoxicity (Fig. 4B), confirming that the observed increase in cell death was mediated specifically through caspase-3/7-dependent apoptosis. These results indicate that DDR inhibition sensitizes cells to Taxol induced apoptosis primarily via the intrinsic executioner caspases.

A


Apoptosis
Sub G1 population
0.0
0.2
0.4
0.6
0.8
*
MCF7 Control
MCF7 Taxol
MCF7-C3 Control
MCF7-C3 Taxol
Control
ATMi 5 μM
DNA-PKi 1 μM
ATRi 0.5 μM
Apoptosis
% of sub-G1
0
20
40
60
*
*
Control
Taxol 0.001 uM
Control
ATMi
DNA-PKi
ATRi
Flow Cytometry
Percent Apoptosis
1
10
100
1000
10000
V79
ATMi
DNA-PKi
ATRi
Without Taxol
With Taxol 0.001 μM

B


Caspase 3/7 activity
Caspase 3/7 (RFU)
0
20000
40000
60000
80000
*
*
*
*
Control
Taxol 0.01 uM
Control
ATMi
DNA-PKi
ATRi
Caspase 3 inhibitor (ac-DEVD-CHO) effect
Survival Fraction
0.0
0.5
1.0
*
*
*
Taxol
Taxol + ATMi
Taxol + DNA-PKi
Taxol + ATRi
Control
ac-DEVD-CHO 100 μM

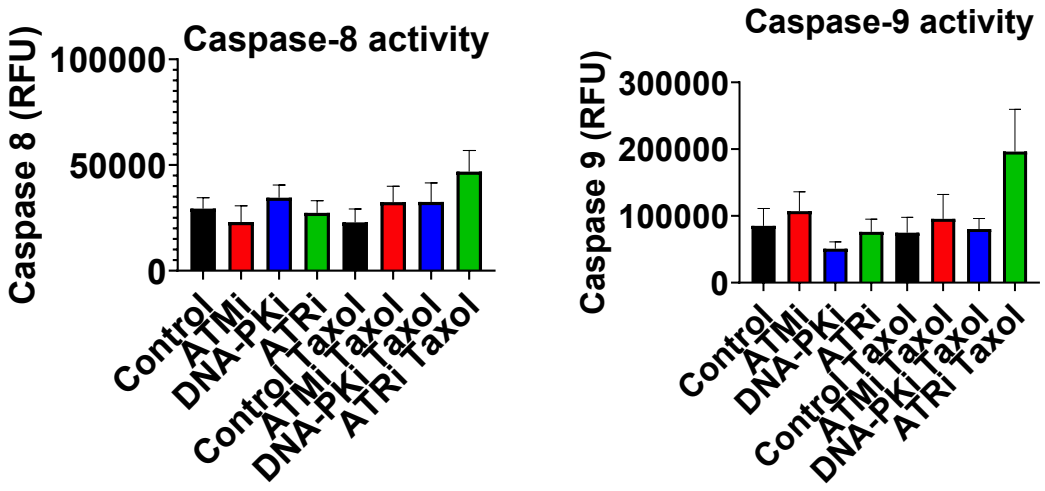


Fig. 4. Cell cycle and apoptosis induction by Taxol and DDR kinase inhibitors.

Cells were treated with 10 nM Taxol and DDR kinase inhibitors (KU55933 (5 μM), VE821 (5 μM), or NU7441 (2.5 μM)) for cell cycle and apoptosis analysis.

A) Apoptotic induction in MCF7 and MCF7-C3 cells after 48 h treatment with 10 nM Taxol and DDR kinase inhibitors: KU55933 (2.5 μM), NU7441 (0.5 μM), or VE821 (0.1 μM). Sub-G1 population after 48 h, measured by flow cytometer

B) Activation of caspase-3/7, caspase-8, and caspase-9 after 48 h, measured by luminometer. Effect of caspase-3 inhibitor on cytotoxicity induced by 2.5 nM Taxol and DDR kinase inhibitors.

Independent experiments were performed at least three times. Error bars indicate SEM. * indicates statistically significant differences ($p < 0.05$).

3.3.5 Prevention of Taxol and DDR inhibitor cytotoxicity by antioxidants in V79 cells

Antioxidants, Naringin and Ascorbyl Glucoside (AA2G) were used in V79 cells alongside Taxol and DDR kinase inhibitors to observe the effects of Taxol on the production of ROS and

oxidative stress. Past research utilized the same antioxidants and saw protection against Taxol and PARP inhibition and saw increased protection which we also expected to find. However, the same concentrations used in this paper with Taxol and DDR inhibitors did not see any protection of the cells. In fact, cell survival decreased in both antioxidants and negative control (V79 cells with Taxol). Almost complete cell death is seen with KU55933 (ATM inhibitor) and NU7441 (DNA-PK inhibitor); however, there is limited cytotoxicity with the use of VE821 (ATR inhibitor). This suggests that the ATR kinase may play a role in antioxidant protection.

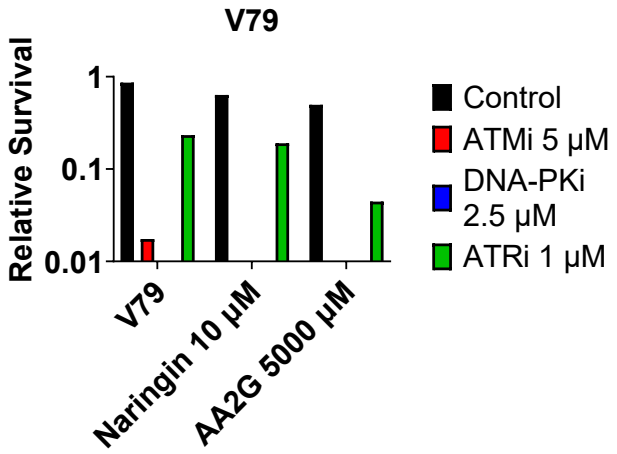


Fig 5. Cell survival of V79 cells with antioxidants, Taxol, and DDR inhibitor treatment

Effect of antioxidants Naringin (10 mM) and Ascorbyl Glucoside (AA2G) 0.5 M with 0.02 uM Taxol and DDR inhibitors.

Independent experiments were performed at least three times. Error bars indicate SEM. * indicates statistically significant differences ($p < 0.05$).

Error bars indicate SEM. * indicates statistically significant differences ($p < 0.05$). Independent experiments were carried out at least three times.

3.4 Discussion

This study revealed a novel relationship between PI3K-like DDR kinases and Taxol-induced apoptosis, mediated primarily through caspase 3/7 activation. We demonstrated that the genetic or pharmacological inhibition of ATM, ATR, or DNA-PK significantly enhances Taxol cytotoxicity in both Chinese hamster and human cancer cells, as shown by reduced proliferation, clonogenic survival and increased sub-G1 populations (Figs. 1–4). Notably, pharmacological inhibition elicited a stronger sensitizing effect than chromic genetic deficiency, suggesting acute kinase inhibition may have additional effects beyond baseline DDR deficiency.

The enhancement of Taxol sensitivity was initially hypothesized to be mediated through checkpoint abrogation, given roles of ATM and ATR in regulating G2/M and S-phase progression. However, both short term and continuous treatment data showed hypersensitization occurred even at low Taxol concentrations that did not induce strong mitotic arrest, and mitotic accumulation was minimal under co-treatment (Fig. 2ABC). These findings indicate that mechanisms beyond classical checkpoint disruption contribute to the observed cytotoxicity. Taxol is known to induce oxidative stress and DNA damage, typically in an S-phase-dependent manner [3]. However, our cytogenetic analyses revealed that DNA damage induced by the combination of Taxol and DDR kinase inhibitors is not limited to S-phase (Fig. 2C).

Chromosomal aberrations were detectable within 3 h of treatment in G2-phase cells. Nonetheless, the frequency of aberrations increased at later points, which corresponded with higher S-phase fractions (Fig. 2D). These observations suggest that while DNA damage can occur outside of S-phase, replication-associated mechanisms likely contribute to the cumulative damage observed with prolonged treatment and this DNA damage would trigger apoptosis. The most consistent and mechanistically informative finding was the dependence of Taxol-DDR inhibitor-induced cell death on caspase 3/7 activation but not on caspase-8 or 9 (Fig. 4C). It

suggests that DDR kinases suppress Taxol-induced caspase-3/7 activation independently of intrinsic or extrinsic apoptotic cascades. Inhibition or genetic deletion of caspase 3 abrogated apoptosis and hypersensitization to Taxol in the presence of DDR kinase inhibitors (Figs. 4D). Notably, MCF7 cells, which lack caspase 3, did not undergo apoptosis under combined treatment. The importance of caspase 3 was confirmed by caspase 3 inhibitor, ac-DEVD-CHO. In contrast, MCF7 cells reconstituted with caspase-3 (MCF7-C3) showed robust induction of apoptosis (Fig. 4D), confirming that caspase 3 is necessary for the observed cell death. This further suggests that other initiator caspase, such as caspase 10 [24], may not be sufficient to mediate apoptosis in this context.

The use of antioxidants was to see if Taxol causes oxidative stress through the production of ROS. Past research saw an increase in protection; however, based on our data, we do not see any increase in cell survival with the addition of Naringin and AA2G antioxidants with Taxol and DDR kinase inhibitors (Fig 5). Though the ATM and DNA-PK inhibitors did not see increased survival, ATR inhibitor VE-821 did see a slight increase in cell survival. This suggests that ATR kinases may play a role in antioxidant protection against oxidative stress. Though we did not see an increase in protection, oxidative stress and ROS production cannot be ruled out due to past research showing protection. Oxidative stress may be a secondary effect of Taxol treatment but is not the main cause of apoptosis as seen in the data.

Although DDR kinase deficiency clearly sensitized cells to Taxol, pharmacological inhibition of DDR kinases induced even greater cytotoxicity (Fig. 3B). This raises the possibility of off-target effects of DDR inhibitors. While KU55933, VE821, and NU7441 are considered selective inhibitors of ATM, ATR, and DNA-PK respectively, they have been reported to target additional PI3K-related pathways. For instance, KU55933 has inhibitory effects on Akt [25],

VE821 can downregulate mTOR signaling [26], and NU7441, although relatively specific for DNA-PK, may also inhibit mTOR and other PI3K family members [11]. Inhibition of these off targets were known to cause sensitization to Taxol [7]. Therefore, it is reasonable to consider that inhibition of these additional PI3K-related targets contributes to the enhanced cytotoxicity observed with Taxol and DDR kinase inhibitors.

Taken together, our findings demonstrate that inhibition of DDR kinases significantly enhances Taxol-induced cytotoxicity via a caspase 3/7-dependent apoptotic pathway, independent of classical cell cycle checkpoint abrogation. This points to a novel therapeutic strategy in which targeting DDR pathways may selectively sensitize tumor cells to Taxol by promoting apoptosis under mitotic stress. Further investigation is warranted to dissect the molecular crosstalk between Taxol, DDR signaling, and apoptotic execution machinery.

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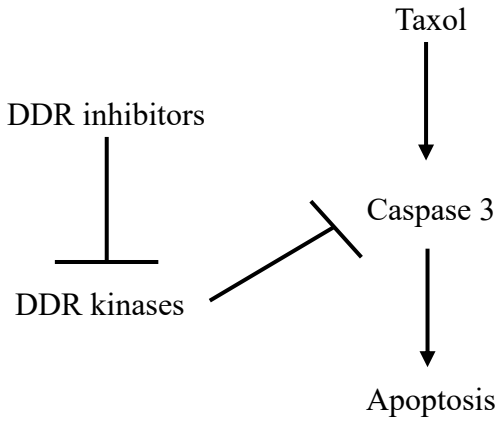
Chapter 4: Conclusion

Based on the data obtained in the figures, our hypothesis that the use of DDR kinase inhibitors would increase Taxol induced cytotoxicity by disrupting DNA repair pathways and activating apoptosis was proven. Taxol causes the cell to arrest in the late G2/M phase; however, the addition of DDR inhibitors does not increase this arrest suggesting the DDR kinases act independently of their cell cycle checkpoint activity. MCF7 and GM5400 cells did not show a decrease in cell survival, unlike V79, V79 mutant, CHO, and U2OS cells. However, the caspase 3 restored MCF7-C3 cells did show an increase in cytotoxicity with Taxol and DDR inhibitors suggesting that Taxol activates caspase 3. This is further seen in the increase in apoptosis via caspase 3 with the addition of Taxol and DDR inhibitors. Caspase 8 and caspase 9 did not show an increase in activity with Taxol and DDR inhibitors suggesting that Taxol bypasses the extrinsic and intrinsic pathway and activates caspase 3 leading to apoptosis. This is further seen with the increase in cell survival with Taxol and DDR inhibitors when caspase 3 is inhibited. The use of antioxidants did not increase cell protection; however, ATR kinases may play a role in antioxidant protection against oxidative stress and ROS formation. Based on the data, oxidative stress is most likely a secondary effect of Taxol induced cytotoxicity. Overall, we can conclude that co-therapy of Taxol and DDR kinase inhibitors increases cytotoxicity in cancer and normal cells through activation of caspase 3/7 leading to apoptosis.

Based on the data, the following model for the mechanism of action of Taxol and DDR kinase inhibitors causing apoptosis is proposed:


Taxol
DDR inhibitors
Caspase 3
DDR kinases
Apoptosis

Chapter 5: Future Directions

This research shows increased cytotoxicity with co-therapy treatment of Taxol and DDR kinase inhibitors. It gave insight into the mechanism of action of Taxol through the activation of caspase 3 and that DDR kinase inhibition prevents DNA repair leading to increased apoptosis. It was confirmed that Taxol stabilizes microtubules and leads to cell cycle arrest. Overall, this research can help give a further understanding of how DDR kinases signal downstream events and how stressors can play a role in the apoptotic pathway. Further research on direct activation of apoptosis could continue our understanding of naturally occurring chemical extract induced cytotoxicity in cancer treatment.

A suggested research potential would be combining PARP inhibition, Taxol, and DDR inhibition to see if cytotoxicity increases further. Since PARP inhibition prevents SSBR, DDR kinase inhibition prevents DSBR, and Taxol activates caspase 3, I expect that a combination would drastically increase cytotoxicity. However, this triple therapy could also drastically increase toxicity clinically. Another co-therapy that needs to be considered is the use of radiation, Taxol, and DDR inhibitors. The use of Taxol and DDR inhibitors could decrease the amount of radiation needed for treatment; however, most clinical radiation treatment is acute while Taxol treatment is chronic. Therefore, split dose radiation therapy may be the best course of action that should be considered for Taxol co-therapy treatment.

Research into more apoptotic mechanisms for Taxol could also be a good route. Due to minimal activation of caspase 8 and 9 in this study, Taxol does not seem to activate caspase 3 through these main caspases in the apoptotic pathway. Looking at other initiator caspases, such as caspase 10 (in the extrinsic pathway), could help improve the understanding of Taxol's mechanism of action. I hypothesize that Taxol caused activation of caspase 3 is through the

activation of alternative apoptotic mechanisms which are activated through other PI3K signals such as AKT or mTOR.

Another potential challenge comes with the clinical use of Taxol and DDR kinase inhibitors as they have the potential for increased cytotoxicity in normal human cells. Though GM5400 normal human fibroblast cells did not see an increase in cytotoxicity with Taxol and DDR kinase inhibitors, this may be due to the way fibroblast cells function in the body through continued repair and creation of the extracellular matrix. Continued research on normal human cell lines with Taxol and DDR inhibition could help the understanding of cytotoxicity in healthy cells and aid clinical research.

Appendix I: Raw Data

Table 1: V79 and inhibitors doubling time

	No Treatment				Taxol 0.01 uM			
Hours	Control	ATMi 5 uM	DNA-PKi 2.5 uM	ATRi 5 uM	Control	ATMi 5 uM	DNA-PKi 2.5 uM	ATRi 5 uM
0	20	20	20	20	20	20	20	20
24	60	74	47	65	60	38	42	29
48	200	138	117	202	346100	191	61	57
72	600	204	866	52	300	663	132	43

Table 2: M1-10 doubling time

	No treatment			Taxol 0.01 uM		
Hours	M110 (1)	M110 (2)	M110 (3)	M110 (1)	M110 (2)	M110 (3)
0	20	20	20	20	20	20
24	117	117	95	65	65	80
48	179	179	225	196	196	166
72	298	298	410	408	408	295

Table 3: XR-V15B doubling time

	No treatment			Taxol 0.01 uM		
Hours	XRV15B (1)	XRV15B (2)	XRV15B (3)	XRV15B (1)	XRV15B (2)	XRV15B (3)
0	20	20	20	20	20	20
24	29	43	29	14	19	14
48	125	58	125	55	35	55
72	224	151	224	135	35	135

Table 4: R1-34 doubling time

	No treatment			Taxol 0.01 uM		
Hours	R134 (1)	R134 (2)	R134 (3)	R134 (1)	R134 (2)	R134 (3)
0	20	20	20	20	20	20
24	52	29	29	40	28	28
48	160	47	47	123	15	15
72	389	212	212	208	169	169

Table 5: pHH3

Control no treatment	Control with Taxol	ATMi no treatment	ATMi with Taxol	DNA-PKi no treatment	DNA-PKi with Taxol	ATRi no treatment	ATRi with treatment

7.7	5.8	6.2	7.1	5.4	6.6	6.8	9.5
2.3	2.8	1.8	4.5	4.1	3.3	2.9	12.8

Table 6: Mitotic Index

Hours after Taxol	Control			ATMi			DNA-PKi			ATRi		
0	0	0	0	0	0	0	0	0	0	0	0	0
3	0.11	0.05	0.07	0.043	0.032	0.033	0.067	0.029	0.064	0.062	0.022	0.047
6	0.15	0.10	0.08	0.094	0.077	0.091	0.14	0.090	0.063	0.088	0.11	0.066
9	0.15	0.19	0.11	0.13	0.12	0.15	0.18	0.15	0.052	0.16	0.13	0.085

Table 7: EdU

Hours after Taxol	V79	ATMi	DNA-PKi	ATRi
0-3	0	0	0	0
3-6	0.33	0.15	0.38	0.52

6-9	0.96	0.90	0.80	0.85

Table 8: Chromosome aberrations

Hours after Taxol	V79	ATMi	DNA-PKi	ATRi
0-3	0	0.1	0.08	0.18
3-6	0	0.25	0.1	0.6
6-9	0	0.15	0.25	0.25

Table 9: V79 and mutants cell survival fractions

Taxol concentration (uM)	V79			M1-10 (ATM -/-)			XR-V15B (Ku80 mutant)			R1-34 (ATR deficient)		
0	1	1	1	1	1	1	1	1	1	1	1	1
2.5	/	/	/	/	/	1	/	1.1	1	1	1	0.7
5	/	/	/	/	/	0.94	0	0.6	0.8	0.7	0.5	/
10	1.1	0.40	1.1	0.8	0.14	0.47	/	0.1	0.6	1	0.3	0
15	/	/	/	0.02	0.09	0.07	/	/	0.1	/	/	0
20	0.36	0.12	0.88	/	/	0	/	0	/	0	/	/
25	0.025	0.20	0.0030	/	/	/	/	/	/	/	/	/
30	0.025	0	0.088	/	/	/	/	/	/	/	/	/

50	0.015	0	0	/	/	/	/	/	/	/	/	/

Table 10: V79 and inhibitors survival fractions

Taxol concentration (uM)	Control			ATMi 5 uM			DNA-PKi 3 uM			ATRi 5 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1
0.5	/	/	/	0.88	1	0.92	0.66	0.40	1.10	0.6	1	0.81
1	/	/	/	/	/	0.57	0.98	0.86	/	/	/	0.95
2.5	/	/	/	/	0.03	0.015	0.01	0.21	0	/	/	0
5.0	/	/	/	0.005	0	0.015	0.01	0	0.01	0.01	0	0
10	0.135	0.228	0.305	/	/	0	0	/	/	/	/	/
20	0.34	0.1	0.53	/	/	/	/	/	/	/	/	/
25	0.025	0.2	0.003	/	/	/	/	/	/	/	/	/
30	0.07	0.01	0	/	/	/	/	/	/	/	/	/

50	0.01 5	0	0	/	/	/	/	/	/	/	/	/

Table 11 CHO cell survival fractions

Taxol concentration (uM)	Control			ATMi 5 uM			DNA-PKi 1 uM			ATRi 5 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1
1	/	/	/	1.1	1.2	1	0.37	0.41	/	0.67	0.14	0.24
2.5	0.79			0.64	0.87	0.84	0.42	0.12	0.24	0.15	0	0.04
5	0.81		0.79	0.065	0.28	0.64	0.007	0	0	0.051	0	0
10	0.59	0.59	0.52	0	0.090	0.3	0	0	/	0.007	0	/
15	/	/	/	/	/	0.3	/	/	/	/	/	/
20	0.44	0.64	0.6	0	/	0.032	0	/	/	/	/	/
30	0.1	0.398	0.25	/	/	/	/	/	/	/	/	/
50	/	0.033	0	/	/	/	/	/	/	/	/	/

Table 12: MCF7 survival fractions

Taxol Concentration (uM)	Control			ATMi 5 uM			DNA-PKi 1 uM			ATRi 0.5 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1

0.01	0.20	0.74	0.47	0.13	0.09	0.83	0.08	0.30	0.46	0.23	0.64	0.06
0.05	0.082	0.54	0.42	0.040	0.03	0.50	0.01	0.16	0.38	0.26	0.47	0.11

Table 13: MCF7-C3 cell survival fractions

Taxol Concentration (uM)	Control			ATMi 5 uM			DNA-PKi 1 uM			ATRi 0.25 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1
0.1	0.99	0.86	0.99	0.67	0.36	0.24	0.65	0.83	0.65	0.35	0.17	0.68
0.2	0.80	0.79	0.52	0.31	0.19	0.18	0.46	0.17	0.20	0.20	0.20	0.28
0.3	0.21	0.31	0.59	0.12	0.12	0.03	0.087	0.096	0.07	0.07	0.03	0.04

Table 14: U2OS survival fractions

Taxol Concentration (uM)	Control			ATMi 1 uM			DNA-PKi 1 uM			ATRi 0.25 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1

0.002	0.66	0.81	0.85	0.67	0.42	0.54	0.33	0.57	0.28	0.68	0.71	0.64
0.01	0.17	0.34	0.42	0.050	0.11	0.19	0.078	0.16	0.056	0.19	0.16	0.088

Table 15 GM5400 survival fractions

Taxol Concentration (uM)	Control			ATMi 2.5 uM			DNA-PKi 0.5 uM			ATRi 0.1 uM		
0	1	1	1	1	1	1	1	1	1	1	1	1
0.001	0.31	0.37	0.51	0.26	0.44	0.32	0.40	0.75	0.64	0.98	0.27	0.58
0.003	0.11	0.32	0.51	0.076	0.27	0.32	0.20	0.26	0.30	0.27	0.29	0.23

Table 16: Sub-G1 population

	No treatment			Taxol 0.001 uM		
Chemical	Total Count	Apoptosis number	% Apoptotic	Total Count	Apoptosis number	% Apoptotic
Control	465000	900	0.19	343000	690	0.20
ATMi 5 uM	648000	14000	2.16	557000	7000	1.26

DNA-PKi 2.5 uM	790000	18000	2.28	285000	49000	17.19
ATRi 1 uM	436000	19000	4.36	329000	50000	15.20

Table 17: MCF7 and MCF7-C3 percentage of apoptotic cells

	Control			ATMi 5 uM			DNA-PKi 1 uM			ATRi 0.5 uM		
MCF-7 Control	0.08	0.1	0.075	0.1	0.17	0.2	0.12	0.2	0.13	0.1	0.07	0.12
MCF-7 Taxol	0.1	0.11	0.14	0.23	0.2	0.25	0.2	0.3	0.22	0.18	0.12	0.13
MCF7-C3 Control	0.2	0.25	0.34	0.25	0.20	0.24	0.3	0.22	0.37	0.28	0.33	0.19
MCF7-C3 Taxol	0.28	0.34	0.22	0.4	0.4	0.43	0.5	0.55	0.43	0.57	0.53	0.42

Table 18: Caspase 3/7 activation

Trial number	Control no treatment	Control with Taxol	ATMi no treatment	ATMi with Taxol	DNA-PKi no treatment	DNA-PK with Taxol	ATRi no treatment	ATRi with Taxol
1	30840	28400	45627	82800	16791	121540	25880	84250
2	2380	25472	5600	54800	8810	69800	16703	19300
3	900	690	14000	7000	18000	49000	19000	50000
4	1700	1100	2900	75000	4300	120000	3200	67000
5	4710	3620	11300	101510	6450	88090	16530	75060
6	7520	10080	5020	27890	16910	19900	9080	122730
7	1630	2420	2740	18010	3850	12820	2250	10370
8	950	1640	1580	14530	2580	9000	1690	8130

Table 19: Caspase 3 inhibition

	Control			ac-DEVD-CHO 100 uM		
Taxol	0.90	0.91	0.88	0.85	0.76	0.90
Taxol + ATMi	0.30	0.35	0.31	0.80	0.73	0.71
Taxol + DNA-PKi	0.25	0.20	0.35	0.55	0.66	0.54
Taxol + ATRi	0.10	0.15	0.12	0.70	0.73	0.77

Table 20: Caspase 8 activation

Trial number	Control no treatment	Control with Taxol	ATMi no treatment	ATMi with Taxol	DNA-PKi no treatment	DNA-PK with Taxol	ATRi no treatment	ATRi with Taxol
1	34000	7500	11000	28000	53000	21000	31000	30000
2	45000	11000	72000	32000	26000	83000	57000	23000
3	10738	8612	6697	35618	9689	19089	7915	37957
4	42207	30268	24864	78683	30108	56656	29400	93068
5	47722	25100	15900	38800	25600	39000	25170	90660
6	19776	6184	4425	10280	23400	6916	4884	39629
7	20410	37100	31460	18220	49970	17810	33920	28550
8	15020	57000	17920	18240	58340	16560	30000	32170

Table 21: Caspase 9 activation

Trial number	Control no treatment	Control with Taxol	ATMi no treatment	ATMi with Taxol	DNA-PKi no treatment	DNA-PK with Taxol	ATRi no treatment	ATRi with Taxol
1	28000	18000	21000	29000	30000	34000	32000	43000
2	46000	39000	49000	29000	39000	47000	32000	39000
3	155810	124303	121269	337909	65113	103203	187682	387294

4	37155	31287	29513	93158	21613	78624	73090	151530
5	51900	214448	156200	106049	59300	172446	91100	277600
6	16668	36346	60199	22428	15980	46900	16000	515686
7	129500	64400	160940	59980	91830	67600	84110	70800
8	216810	71140	258700	87590	85150	93880	92510	85990

Table 22 V79 antioxidant survival fractions

	Control			ATMi 5 uM			DNA-PKi 2.5 uM			ATRi 1 uM		
Taxol 0.02 uM	0.64	1	1	0.053	0.030	0.0033	0.020	0	0.0067	0.11	0.44	0.26
Naringin + Taxol 0.02 uM	0.52	0.85	0.57	0.037	0.0067	0	0.020	0	0	0.093	0.45	0.16
AA2G + Taxol 0.02 uM	0.33	0.80	0.46	0.0033	0.0033	0.0033	0.0067	0	0	0.017	0.26	0.020