의학박사 학위논문

Antitumor Activity of ATR inhibitor AZD6738 in Breast Cancer Cells

유방암 세포주에서 ATR 억제제를 이용한 항종양 효과

2014년 6월

서울대학교 대학원
의학과 분자종양의학 과정
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Antitumor Activity of ATR inhibitor AZD6738 in Breast Cancer Cells

By

Hee Jun Kim

(Directed by Seock-Ah Im, M.D., Ph.D.)

A Thesis Submitted to the Interdisciplinary Graduate Program in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Science (Molecular and Clinical Oncology) at the Seoul National University, Seoul, Korea

June, 2014

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ABSTRACT

Antitumor Activity of ATR inhibitor AZD6738 in Breast Cancer Cells

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Background: The DNA repair system is critical for maintaining genomic integrity. Defects in the DNA damage repair (DDR) pathway can lead to chromosomal aberrations resulting in cancer with genetic instabilities. Recent findings suggest that

competent DNA repair in the cancer cell induces resistance to therapeutic agents. Defects in the DDR pathway are common in high grade cancer patients. Therefore, the inhibition of DNA repair could lead to reduce the drug resistance and induce the accumulation of errors which is becoming an attractive strategy for cancer treatment. DNA damage response usually starts with the "sensing" or "detection" of the DNA damage to maintain genomic integrity. The ataxia telangiectasia and Rad3-related (ATR) can be activated by various types of DNA damage and it initiates DNA damage-induced signalling cascade. ATR is a master regulator of DDR, signaling to control cell cycle transitions, DNA replication, DNA repair, and apoptosis. Therefore, the ATR pathway might be useful target for new drug development and it is important that the effects of many current cancer treatments are modulated by DDR.

Materials and Methods: We studied the growth inhibitory effects of AZD6738 on

human breast cancer cell lines using MTT assay. Cell cycle analysis and western blotting were also performed to determine molecular changes induced by AZD6738. Immunofluorescence assay and comet assay were conducted to understand the action mechanisms of AZD6738 on breast cancer cells.

Results: Anti-proliferative effects and the inhibition of DDR activity by ATR inhibitor, AZD6738 on human breast cancer cell lines were explored in this study. MTT assay resulted in the heterogenous response. AZD6738 induces cell cycle arrest and apoptosis in breast cancer cell lines. AZD6738 impaired DNA damage repair function and promoted cell death by damage accumulation in sensitive cells. In sensitive cell line, SKBR-3, the expression of phosphorylated CHK1 (S345) was downregulated with the other DNA repair molecules; RAD51, MRE11 and ERCC1 as opposed to less sensitive breast cancer cell BT-474. The number of RAD51 foci was significantly

decreased at the sites of DNA damage in SKBR-3 after AZD6738 treatment. The decreased functional CHK1, which interacted with RAD51 foci, leads to the accumulation of DNA damage due to HR inactivation. And it was also identified that ATR inhibitor potentiate the efficacy of cytotoxic chemotherapeutic agents, cisplatin and paclitxel in breast cancer cell line.

Conclusion: Understanding the antitumor efficacy and the mechanisms of ATR inhibitor in the breast cancer cell lines open up the possibility of future clinical trial targeting DNA damage repair in breast cancer.

***Keyword*: DNA damage response, ATR inhibitor, Homologous recombination**

***Student Number*: 2011-31143**

TABLE OF CONTENTS

ABSTRACT i

TABLE OF CONTENTS v

LIST OF TABLES vi

LIST OF FIGURES vii

INTRODUCTION 1

PURPOSE OF THIS STUDY 5

MATERIALS AND METHODS 6

RESULTS 12

DISCUSSION 33

CONCLUSIONS 38

REFERENCES 39

ABSTRACT IN KOREAN 44

LIST OF TABLES

TABLE 1. IC_{50} value of AZD6738 in breast cancer cell lines …… …. …. 14

TABLE 2. Baseline protein levels of DDR molecules in human breast cancer cell lines

…… 15

TABLE 3. Combination of AZD6738 and cisplatin shows synergism in some breast

cancer cells. …… 31

TABLE 4. Combination of AZD6738 and paclitaxel shows synergism in some breast

cancer cells …… 32

LIST OF FIGURES

Figure 1. Antiproliferative effect of AZD6738 : AZD6738 effectively suppresses cell viability in some human breast cancer cells ····· 13

Figure 2. Cell cycle analysis and Western blot analysis of PARP and cyclin E : AZD6738 induces S phases cell cycle arrest and apoptosis in SKBR-3 sensitive cell line. ATR inhibitor induced PARP cleavage and downregulation of cyclin E in SKBR-3 cells ····· 17

Figure 3. Flow cytometric analysis using propidium iodide and Annexin V-FITC : AZD6738 induces apoptosis in SKBR-3 sensitive cell line.. ····· 18

Figure 4. Western blot analysis of key DDR molecules : ATR inhibition effects on DDR pathway. SKBR-3 showed a decrease in the expression of CHK1

phosphorylation along with a reduction in DNA repair markers such as RAD51, MRE11, ERCC1 ····· 20

Figure 5. Western blot analysis of key signaling molecules : AZD6738 inhibits proliferative signaling. SKBR-3, sensitive cell line, showed a decrease in the expression of p-AKT and p-ERK, whereas increased in BT-474 in dose–dependent manner ····· 21

Figure 6. ATR inhibition impairs RAD51 foci formation during the DNA damage response ····· 24

Figure 7. Comet assays : ATR inhibition leads to accumulation of DNA damage in SKBR-3 but not in BT-474 ····· 25

Figure 8. The levels of nuclear CHK1 expression determines the efficiency of HR DSB repair ····· 28

INTRODUCTION

A poly (ADP-ribose) polymerase (PARP) inhibitor is a rationally designed targeted therapy for cancers with impaired DNA repair abilities; breast and ovarian cancer patients harboring mutation in BRAC1 or BRCA2 gene (1, 2). Only 5-10% of breast cancer cases are thought to be caused by germ-line mutation (3, 4). The majority of breast cancers are sporadic breast cancer which has an acquired mutation or epigenetic inactivation within genes involved in DNA damage repair (5, 6). Breast cancers are intimately related to DNA damage repair defects or defects in cell-cycle checkpoints which allow damaged DNA to go unrepaired (7). Both ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) and check-point kinase 1 (CHK1) have been proposed as potential breast cancer susceptibility genes. A small number of gene-based association studies have been carried out to investigate the SNPs of ATR-CHK1 DNA damage pathway in association with breast cancer risk (8, 9). It is estimated that these breast cancer cells have enhanced DNA damage repair activity which confers a survival advantage.

DNA-damaging agents represent the cornerstone for the treatment of tumors, yet for the majority of patients they provide only modest benefit. Several

cytotoxic agents including taxane and anthracycline have been the standard therapeutic regimen in breast cancer (10, 11). Recently studies of BRCA1-associated breast cancers show that these cancers are sensitive to DNA-damaging agents such as platinum drugs and PARP inhibitor olaparib (12-15). Many anti-cancer drugs induce DNA damage and activate cellular responses, such as DNA damage checkpoint signaling cascades, resulting in cell cycle arrest at the G1/S, intra-S and G2/M phases. This allows time for damage repair, or leads to apoptosis when the extent of DNA damage is not compatible with cell survival (16, 17). Thus, DNA damage response (DDR), which prevents the passage of damaged DNA to the next generation of cells, can hamper tumor progression by inducing tumor cell death in patients undergoing chemo/radiotherapies (18). DDR is largely regulated by the phosphoinositol 3-kinaselike serine/threonine protein kinases (PI3Ks), ATM and ATR (19-21). The ATM activation is associated with ionizing radiation and double strand break (DSB). In contrast, ATR is activated by single-stranded DNA (ssDNA), which can occur at persistent DSB, but more extensively on stalled replication fork (19). ATM and ATR share a number of substrates, such as protein p53, but there are also substrates specific for each kinase - CHK1 is regulated by ATR, while CHK2 is an ATM substrate (22, 23). In contrast to ATM or CHK2, ATR and CHK1 are essential in mammals, which have placed limitations on functional

studies in this pathway. As a sensor of altered DNA structures, ATR is activated by many of the most commonly used antiproliferative agents used for cancer therapy.

ATR signaling in turn activates downstream pathways that control cell-cycle arrest and mediate cell survival (24). The activation of ART occurs only in S- to G2 phase (25). ATR phosphorylates CHK1 on S317; this modification is required for the phosphorylation of neighboring CHK1 sites, including S345, and the resulting activation of the G2-M checkpoint (26). Wan et al. showed that a significant fraction of CHK1 is phosphorylated following DNA damage induced by a variety of agents including UV lights, ionizing radiation in an ATR dependent manner (27).

Oncogene-induced replication stress, activation of HER2, activates the ATR pathway explaining high levels of DDR activation in many neoplasias (28, 29). Previous studies showed that HER2 expression modulated the repair of specific DNA lesion produced by chemotherapy (30). These studies have suggested that HER2 overexpression facilitates DNA repair mechanisms, resulting in decreased cytotoxic effects to most cancer chemotherapeutic agents. PI3K is a downstream molecule of the HER2 signaling pathway, and PI3K mutation leads to stimulation of AKT axis (31). The increased DNA repair activity by AKT activation has caused the resistance of antitumor therapy and treatment failure.

In such a cancer treatment, ATR inhibitor as a DNA repair inhibitor to increase the DNA damaging effect could be a new strategy for the treatment of HER-2 positive breast cancer patients.

As conventional anticancer treatments are toxic to cells irrespective of whether they are normal or cancerous, there have been efforts for sensitizing cancer cells in order to enhance anticancer treatment efficacy. (32, 33). Although several ATR inhibitors have been reported (34), a specific compound that inhibits ATR protein kinase has yet to be discovered. So I investigated the antitumor activity of ATR inhibitor, AZD6738 in breast cancer cell lines *in vitro*. In this study, the effect of ATR inhibition on DNA repair *in vitro* was examined in breast cancer cell lines. The antitumor effect and action mechanism of ATR inhibitor as a mono-agent was determined by using BT-474 and SKBR-3 cell line which sensitivities had been found different to ATR inhibitor. At the same time, the role of ATR inhibitor as a chemosensitizer was also identified through the combination with cytotoxic chemotherapeutic agents. This is the first report to show that HER2 positive breast cancer cells are selectively sensitive to ATR inhibitor, AZD6738 depending on the PI3K mutation by S phase cell cycle arrest and apoptosis. These data warrant further evaluation of AZD6738 in future clinical trials.

PURPOSE OF THIS STUDY

1) To investigate the growth inhibitory activity of AZD6738 in breast cancer cell lines

2) To understand the molecular basis of growth inhibition

3) To identify the action mechanism of AZD6738

4) To investigate the role as a chemosensitizer through the combination with cytotoxic chemotherapeutic agents.

MATERIALS and METHODS

1. Reagents

AZD6738 was kindly provided by AstraZeneca (Macclesfield, Cheshire, UK). The compound was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 4.125 mg/mL. Cisplatin and paclitaxel were obtained from Choongwoe Co., Ltd. and Samyang Genex Co., Ltd. Aliquots of the solution were then stored at -80℃.

2. Cell lines and cell culture

Human breast cancer cells (MCF-7, T47D, HCC-70, HCC-1143, MDA-MB-157, MDA-MB-231, BT-549, MDA-MB-468, Hs578T, HCC-1937, MDA-MB-453, BT-474, and SKBR-3) authenticated using short tandem repeat analysis were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were banked and passaged for less than 6 months before use, and they were cultured in RPMI 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% FBS (WELGENE Inc., South Korea) and 10 μg/mL gentamicin (Cellgro, Manassas, VA, USA) at 37℃ in a 5% CO_2 atmosphere.

3. Cell growth inhibition assay

Cell (2-3 x 10^3 in 100μL/well) were seeded in 96-well plates and incubated overnight at 37℃ in 5% CO_2. The cells were exposed to increasing concentrations of AZD6738, cisplatin, paclitaxel, or AZD6738 plus either of chemotherapeutic agents for 5 days. After drug treatment, 50μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide solution (Sigma Aldrich) was added to each well and the plates were incubated for 4 hours at 37℃ before the media was removed. After dissolving the formazan crystals with 150μL of DMSO, the absorbance of each well was measured at 540 nm with a VersaMax^{TM} microplate reader (Molecular Devices; Sunnyvale, CA, USA). The absorbance and IC_{50} of AZD6738 were analyzed using SigmaPlot software (Statistical Package for the Social Sciences, Inc. (SPSS); Chicago, IL, USA). Six replicate wells were included in each analysis and at least three independent experiments were conducted.

To evaluate the effects of AZD6738 administered in conjunction with other chemotherapeutic agents (cisplatin or paclitaxel), the cells were treated with serial dilutions of each drug alone or with a combination of AZD6738 with either chemotherapeutic agent at a fixed ratio corresponding to the specific IC_{50} of each drug. After 120 hours of drug exposure, cell proliferation was measured using an MTT assay as described earlier. Any synergistic effects resulting from

co-treatment with the compounds were measured using the methods described by Chou and Talalay. Analysis of the median effect was conducted using Calcusyn software (Biosoft) to determine the combination index values (CI > 1: antagonistic effect, CI = 1: additive effect, and CI < 1: synergistic effect).

4. Western blot analysis

Cells were collected after drug treatment, washed with ice-cold PBS, and incubated in extraction buffer [50mM Tris-CI(pH7.4), 150mM NaCI, 1% NP40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride, 1 mg/mL pepstatin A, 0.2 mM leupeptin, 10 ㎍/mL aprotinin, 1 mM sodium vanadate, 1 mM nitrophenylphosphate, and 5mM benzamidine] on ice for 30 min. The lysates were cleared by centrifugation at 13,000 rpm for 20 min. Equal amounts of proteins were separated on an 8%-15% SDS-polyacrylamide gel. The resolved proteins were transferred onto nitrocellulose membranes, and the blots were probed with primary antibodies overnight at 4℃. Antibodies against p-STAT3, STAT3, p-AKT, AKT, p-ERK, ERK, XRCC1, MRE11(31H4), p-ATR(ser428), ATR, p-CHK1(133D3, D12H3), and caspase-3 were purchased from Cell Signaling Technology (Beverley, MA, USA), and anti-RAD51 (H-92) was obtained from Santa Cruz Biotechnology (Santa Cruz;

CA, USA). Anti-phosphorylated histone H2AX (clone JBW301, Millipore; Billerica, MA, USA) and anti-PARP (BD Biosciences; Bedford, MA, USA) were also purchased. Actin antibody (Sigma Aldrich) was used as a control. Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer's protocol (Amersham Biosciences; Piscataway, NJ, USA).

5. Cell cycle analysis

Cells treated with AZD6738 were harvested, fixed with cold 70% ethanol, and then stored at -20℃ for at least 24 hours. The cells were washed in PBS and incubated with 10 ㎍/mL RNase A(Sigma Aldrich) at 37℃ for 20min. Next, the cells were stained with 20 ㎍/mL propidium iodide (Sigma Aldrich) and the DNA contents of the cells (10,000 cells per experimental group) were quantified using a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences; San Joes, CA, USA)

6. Annexin V–binding assay for apoptosis

After the cells were exposed to AZD6738, the degree of apoptosis was assessed using the Annexin V–binding assay according to the protocols of the manufacturer (BD PharMingen). The harvested cell suspension was then

incubated with Annexin V for 15 minutes at room temperature in the dark and then analyzed by flow cytometry.

7. Immunofluorescence assay (IFA, RAD51 foci formation)

Cells were plated on 0.01% poly-L-Lysine (Sigma Aldrich)-coated coverslips, and treated with 1 μmol/L of AZD6738 or 5μmol/L of hydroxyurea (HU) or AZD6738 plus HU for 5 days. Afterwards, the coverslips were rinsed once in PBS (37℃), fixed in 3.7% paraformaldehyde for 10 min, permeabilized with PBS-T (0.5% Triton X-100 in PBS) for 5 min, and incubated with primary antibody for 24 hours at 4℃. The primary antibodies used in this study were rabbit polyclonal anti-RAD51 (H-92, Santa Cruz Biotechnology) and mouse monoclonal anti-phosphorylated histone H2AX (clone JBW301, Millipore) at a dilution of 1:50. The coverslips were rinsed three times for 10 min in PBS followed by incubation with the appropriate fluorophore-conjugated secondary antibody (Invitrogen; Carlsbad, CA, USA). The cells were counterstained with DAPI (300 nM; Invitrogen) and the coverslips were mounted on slides using Faramount aqueous mounting medium (DAKO; Denmark). Immunofluorescence was visualized using a Zeiss LSM 510 laser scanning microscope.

8. Comet assays

Cells were treated with 1 μmol/L AZD6738 for 5 days. After treatment, cells were trypsinized and subjected to an alkaline comet assay using the Trevigen Comet assay kit (Trevigen; Gaithersburg, USA) following the manufacturer`s protocol. Tail lengths were measured with Comet assay Ⅳ program.

9. Statistical analysis

Statistical analyses were performed using SigmaPlot version 9.0. A two-sided Student's t-test was used when appropriate. The results are expressed as the mean ± SD or ± SE. A P-value <0.05 was considered to be statistically significant.

RESULTS

Anti-proliferative effects of AZD6738 on breast cancer cell lines

Cell survival following the treatment with AZD6738 was measured using a MTT assay to evaluate the effect of ATR inhibitor in breast cancer cell lines. ATR inhibition by AZD6738 resulted in the heterogenous response among the breast cancer cells. The IC_{50} values were determined ranging from 0.3 to higher than 1 μmol/L (Fig. 1, Table 1). To find the predictive marker, the baseline protein levels of DDR molecules in human breast cancer cell lines were measured by western blotting. The protein levels of ATM, ATR, MRE11 and CHK1 were expressed to varying degrees in the panel of breast cancer cell lines and RAD51 and PARP were expressed to similar degrees in all cell lines (Table 2). A correlation between the anti-proliferative effect of AZD6738 and the levels of protein expressions well known as predictive markers for DDR inhibitors was not found. Since results of MTT showed different sensitivity to AZD6738 in spite of the same HER2 + subtype, two cell lines SKBR-3 (IC_{50} =0.59±0.08) and BT-474 (IC_{50} >1 μmol/L) were chosen for further study.

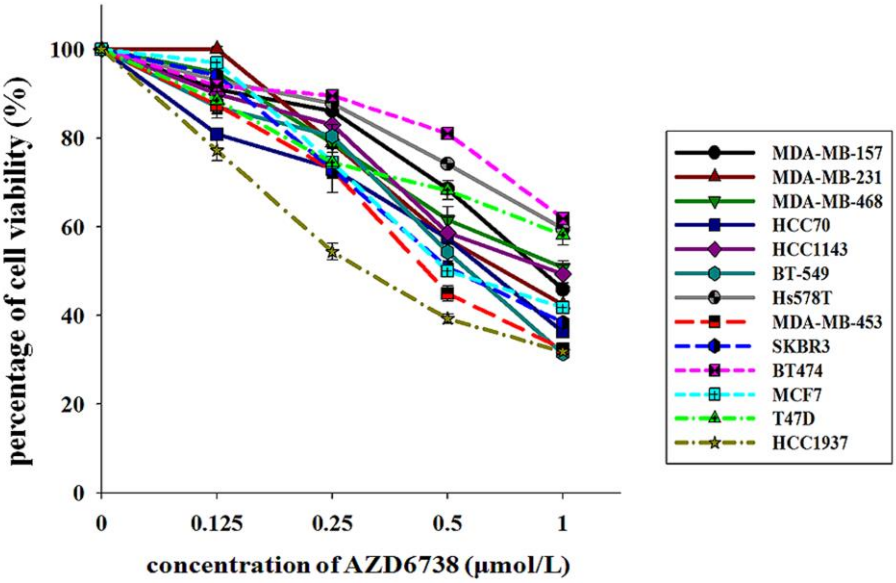


Figure 1. Antiproliferative effect of AZD6738 : AZD6738 effectively suppresses cell viability in some human breast cancer cells

Table 1. IC_{50} value of AZD6738 in breast cancer cell lines

Cell line	subtype	TP53	mutation	IC_{50} (μmol/L)	
MDA-MB-157	TNBC	mt		0.91±0.2	Moderate
MDA-MB-231	TNBC	mt	K-ras mt, CDKN2A mt	0.74±0.13	Sensitive
MDA-MB-468	TNBC	WT	PTEN mt, Rb mt	0.98±0.03	Moderate
HCC70	TNBC	mt	PTEN mt	0.61±0.028	Sensitive
HCC1143	TNBC	mt	PIK3CA mt	0.90±0.09	Moderate
BT-549	TNBC	WT	PTEN loss	0.62±0.12	Sensitive
Hs578T	TNBC	mt	CDKN2A mt	>1	Insensitive
HCC1937	TNBC	WT	BRCA1 mt	0.32±0.06	Sensitive
MDA-MB-453	HER2+	WT	PIK3CA mt	0.47±0.17	Sensitive
SK-BR-3	HER2+	WT	PIK3CA mt	0.59±0.08	Sensitive
BT-474	HER2+	WT		>1	Insensitive
MCF-7	Luminal	WT	PIK3CA mt	0.64±0.07	Sensitive
T-47D	Luminal	mt	PIK3CA mt	>1	Insensitive

TNBC, triple negative breast cancer; WT, wild type; mt, mutation.

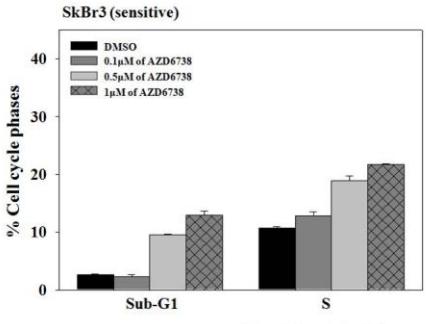
Table 2. Baseline protein levels of DDR molecules in human breast cancer cell lines

Breast Ca.	subtype	ERα	ERβ	PR	AR	HER2	ATM	ATR	Mre11	Chk1	RAD51	ERCC1	TP53	EGFR	mutaion
MDA-MB-157	post-EMT	-	+	-	+	Normal	High	High	Low	Low	Medium	Low	mt		
MDA-MB-231	post-EMT	-	+	-	+	Normal	High	High	High	High	High	Medium	mt		
MDA-MB-468	basal like	-	+	-	+	Normal	Medium	Low	Medium	High	High	Low	WT	↑↑	PTEN mt
HCC70	basal like	-	+	+	+ (weakly positive)	Normal	High	Low	High	High	High	High	mt		PTEN mt
HCC1143	basal like	-	+	-	+ (stongly positive)	Normal	Medium	High	High	Low	High	Medium	WT		
BT-549	post-EMT	-	+	-	+ (stongly positive)	Normal	High	High	High	High	High	Medium	WT		PTEN loss
Hs578T	post-EMT	-	+	-	+	Normal	High	High	High	High	High	Medium	mt		
MDA-MB-453	HER2	-	+	-	+ (stongly positive)	Amplified	Medium	Low	High	High	High	High	–		
SKBr 3	HER2	-	+	-	+ (weakly positive)	Amplified	Low	Medium	High	High	High	Medium	WT		
BT-474	HER2	+	+	+	+ (stongly positive)	Amplified	NA	NA	NA	NA	NA	NA	WT		PI3K mt
MCF 7	luminal(ER)	+	+	-	+ (weakly positive)	Normal	Low	Low	Medium	Low	High	Low	WT		PI3K mt
T47D	luminal(ER,PR)	+	+	+	+ (stongly positive)	Normal	High	High	High	High	High	High	mt		
HCC1937	post-EMT	-	NA	NA	NA	Normal	High	Medium	Low	High	High	Medium	WT		BRCA 1 mt

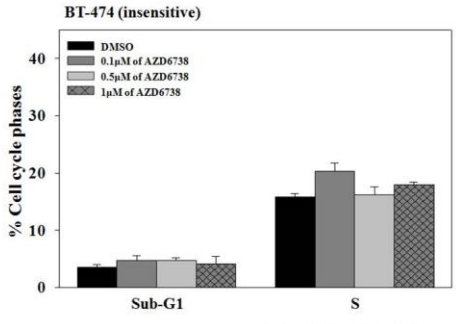
AZD6738 induces cell cycle arrest and apoptosis

ATR is involved in sensing DNA damage and activating the DNA damage checkpoint, leading to cell cycle arrest. We therefore investigated whether AZD6738 promotes cell cycle arrest and/or apoptosis in human breast cancer cells by performing a FACS analysis after 5 days AZD6738 exposure on indicated concentrations. The treatment of AZD6738 led to growth arrest in the S phase of the cell cycle in SKBR-3 cells and increased populations of apoptotic cells were also detected (Fig.2A). The relative expression levels of cell cycle related proteins were investigated by western blot. As with the FACS results, AZD6738 treatment induced expressions of cleaved PARP in sensitive cell lines and the reduction of cyclin E was also observed in SKBR-3 cells (Fig.2B). To evaluate the apoptotic effects of ATR inhibition, we conducted flow cytometric analysis using propidium iodide and Annexin V-FITC (Fig. 3). The percentage of sub-G1 and Annexin V positive/PI negative cells was increased significantly after AZD6738 treatment in sensitive cell line, SKBR-3 cells compared with ATR inhibition in BT-474 cells (Fig. 3).

(A)



Sub-G1 phase (%)	2.6	2.3	9.55	13
S phase (%)	10.75	12.8	18.9	21.75



Sub-G1 phase (%)	3.60	4.78	4.76	4.09
S phase (%)	15.80	20.32	16.18	17.95

(B)

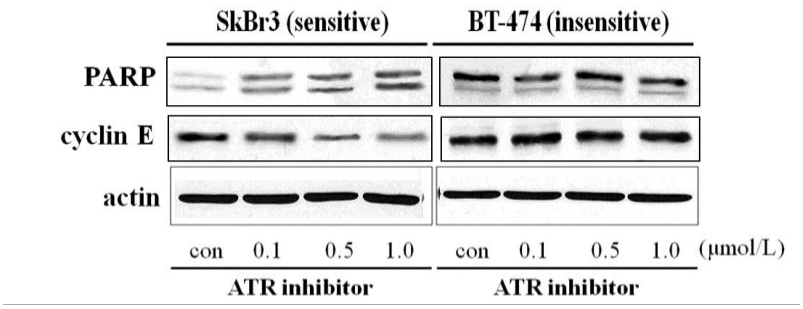


Figure 2. Cell cycle analysis and Western blot analysis of PARP and cyclin E : AZD6738 induces S phases cell cycle arrest and apoptosis in SKBR-3 sensitive cell line. ATR inhibitor induced PARP cleavage and downregulation of cyclin E in SKBR-3 cells

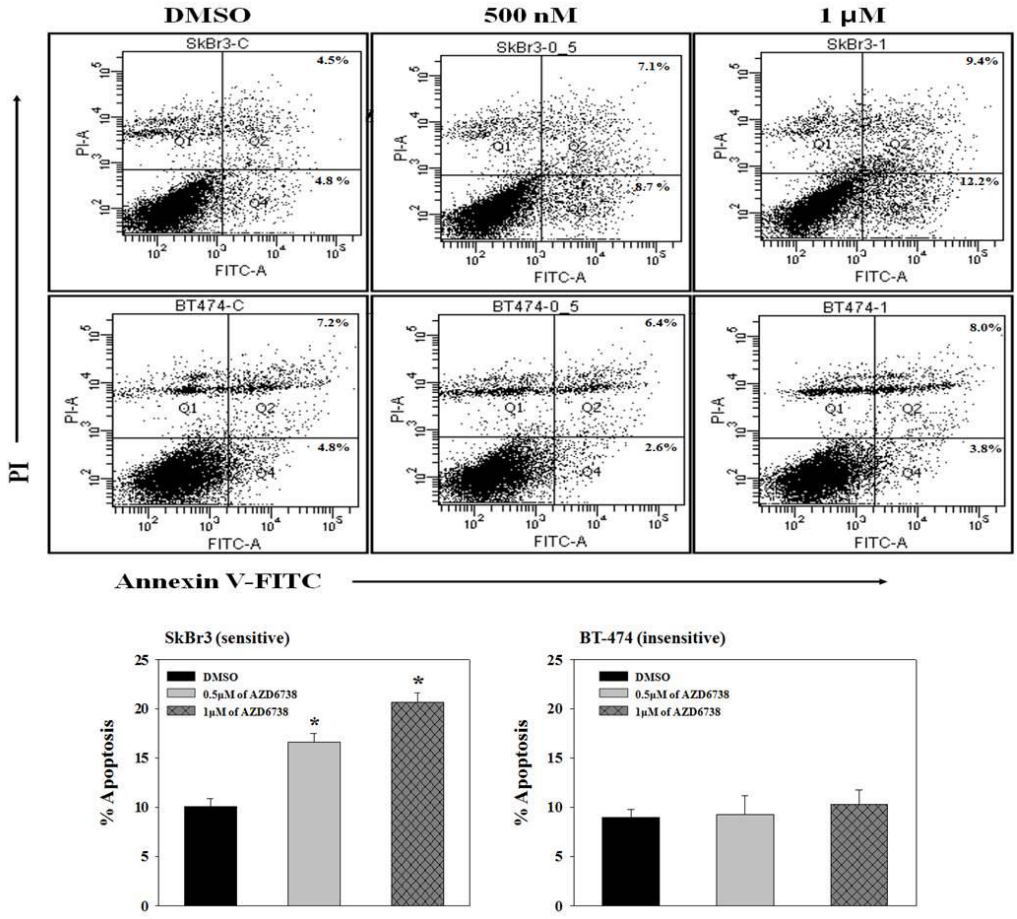


Figure 3. **Flow cytometric analysis using propidium iodide and Annexin V-FITC :** AZD6738 induces apoptosis in SKBR-3 sensitive cell line.

The effect of AZD6738 on DDR pathway in human breast cancer cells

I next evaluated how inhibition of DDR activity by AZD6738 affects homologous recombination repair activities. Cells were exposed to increased concentrations of AZD6738 for 5 days and then subjected to western blot of XRCC1, RAD51, ERCC1, ɣ-H_2AX and phosphorylated CHK1 (Fig. 4). SKBR-3 showed a decrease in the expression of CHK1 phosphorylation along with a reduction in DNA repair markers such as RAD51, MRE11, ERCC1. On the contrary, ɣ-H_2AX as an indicator of DNA damage, was increased. In sensitive cell line SKBR-3, inhibition of ATR resulted in an inhibition of HR repair in dose-dependent manner. However, BT-474 cells showed no significant change on HR molecules by ATR inhibitor using AZD6738. Recent report suggested that ATR is preferentially important for proliferation. We thus checked inhibition of proliferative signaling resulted in an indirect inhibition of AKT, STAT3, and MAPK (Fig. 5). Western blot of SKBR-3, showed a decrease in the expression of p-AKT and p-ERK in a dose-dependent manner, whereas increased in BT-474 after the treatment of ATR inhibitor.

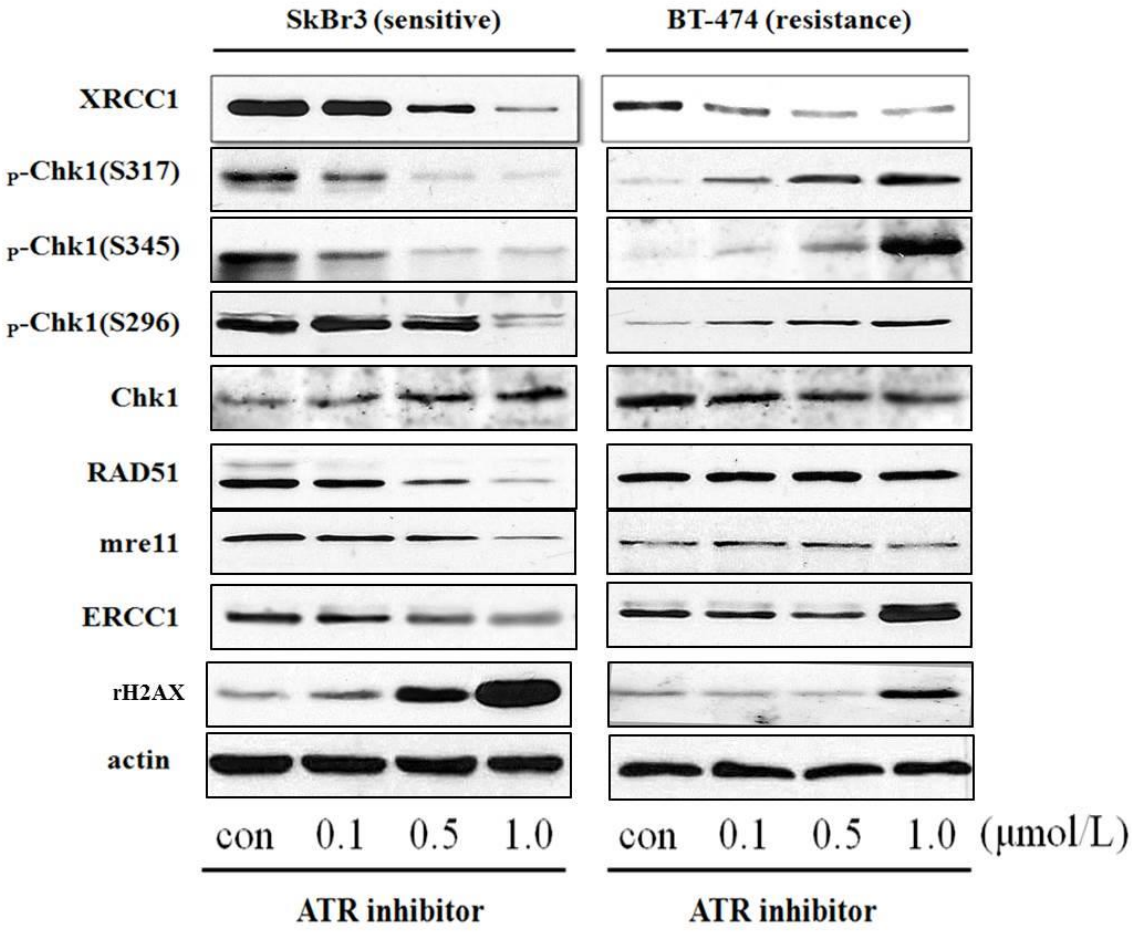


Figure 4. Western blot analysis of key DDR molecules : ATR inhibition effects on DDR pathway. SKBR-3 showed a decrease in the expression of CHK1 phosphorylation along with a reduction in DNA repair markers such as RAD51, MRE11, ERCC1.

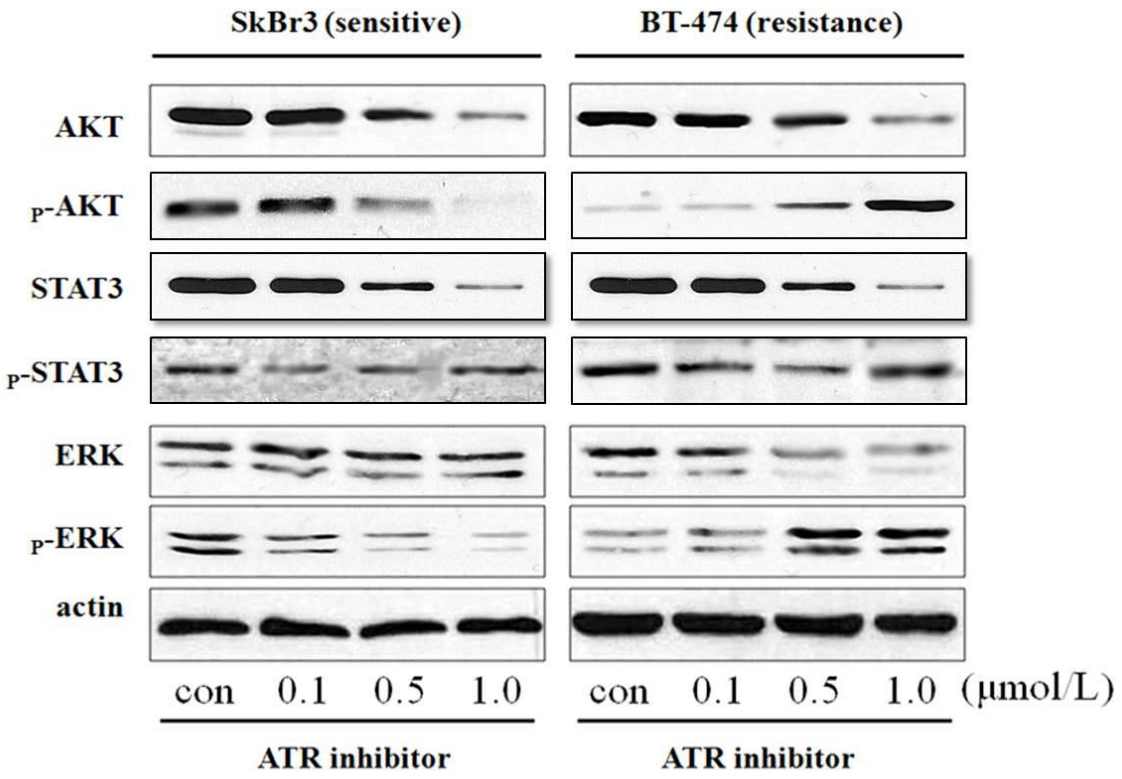


Figure 5. Western blot analysis of key signaling molecules : AZD6738 inhibits proliferative signaling. SKBR-3, sensitive cell line, showed a decrease in the expression of p-AKT and p-ERK, whereas increased in BT-474 in dose –dependent manner

ATR inhibition impairs the efficiency of HR DSB repair

I hypothesize that AZD6738 sensitivity results from reduced HR repair efficiency of ATR inhibition-induced DSBs. The effect of AZD6738 treatment on DDR pathway was identified by the western blot. (Fig. 4) Western blot of sensitive cell lines, SKBR-3, showed a decrease in the expression of CHK1 phosphorylation, which the substrate of ATR, along with a reduction in DNA repair markers such as RAD51, MRE11, ERCC1. Therefore, it was confirm that cell cycle arrest and apoptosis were due to impairment of DNA damage repair function. I performed an immunofluorescence study to examine RAD51 foci formation, indicative of DNA repair activity in cells exposed to AZD6738 and/or hydroxyurea (HU). The number of RAD51 foci was significantly reduced at the sites of DNA damage (ɣ-H_2AX positive) after AZD6738 treatment in sensitive cells even when the degree of damage inducing by HU was comparable to that of the insensitive cells, BT-474 (Fig. 6). A comet assay was performed to ascertain if the decreased molecules of DDR induced accumulation of DNA damage (Fig. 7). SKBR-3 cells showed accumulation of DNA DSBs when treated with AZD6738 whereas BT-474 showed decreased accumulation. RAD51 foci (marker of HR activity) was additionally investigated whether this accumulation of DNA was correlated with decreased

capacity of DDR. These data support that the decreased ability of HR induced the accumulation of DNA damage by ATR inhibitor treatment.

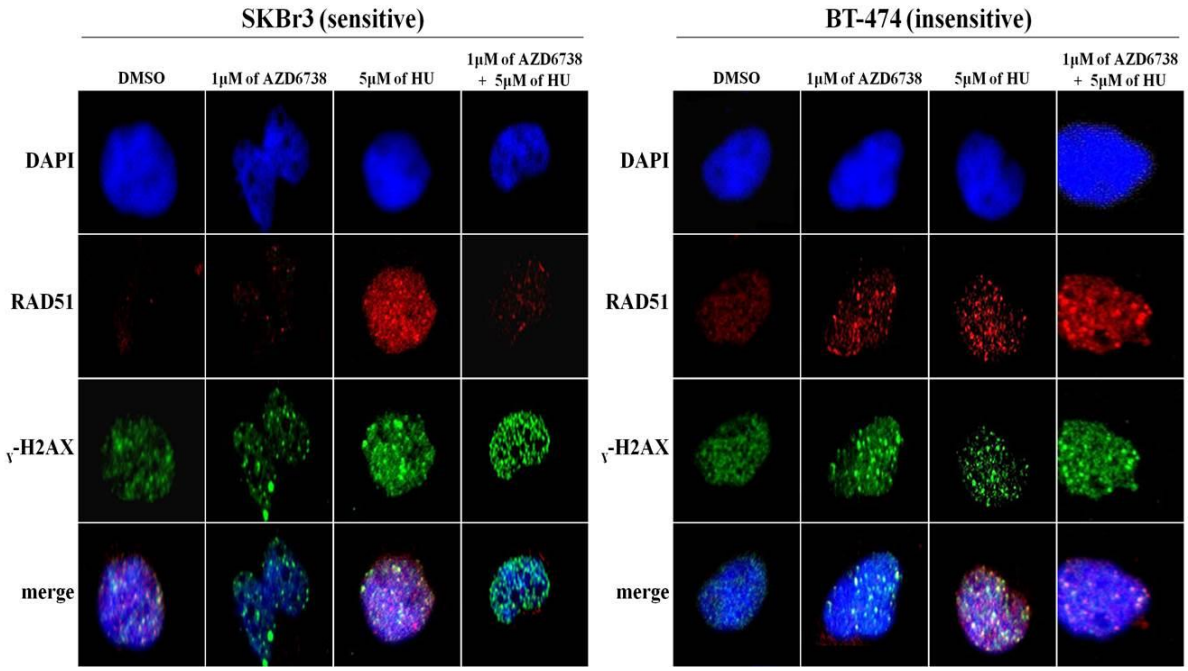


Figure 6. Immunofluorescence assay (IFA, RAD51 foci formation) : ATR inhibition by AZD6738 impairs RAD51 foci formation during the DNA damage response in sensitive SKBR-3 cells

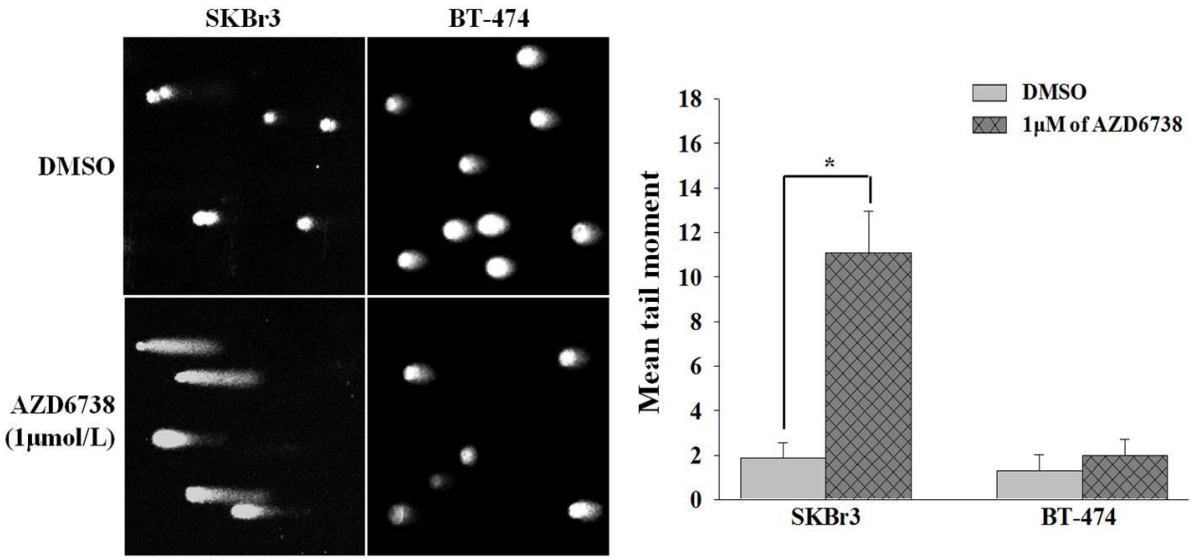


Figure 7. Comet assays : ATR inhibition leads to accumulation of DNA damage in SKBR-3 but not in BT-474.

ATR inhibition disrupts functional localization of CHK1 resulting in HR inactivation

Figure 4 shows phosphorylation of CHK1 at serine 345 (S345) was decreased by ATR inhibitor treatment in SKBR-3, whereas increased in BT-474. It is important that CHK1 is phosphorylated on S345 in response to DNA damage. Therefore, IFA was performed to test whether a reduction in nuclear localization of CHK1 was induced by the decreased phosphorylation on S345 of CHK1 by ATR inhibition. A phosphorylation on S345 leads to nuclear localization of CHK1 protein (26, 35). Phosphorylation of CHK1 serine 345 correlated with an increment of functional CHK1 and co-localization with RAD51 as well as translocation to nucleus, in DDR (35). Figure 8A shows a decrease in the nuclear expression of CHK1 in SKBR-3, and an increase in BT474 in a dose-dependent manner of AZD6738. The nuclear expression of CHK1 in SKBR-3 decreased, whereas increased in BT-474 under the ATR inhibition condition with DNA damaged at the fraction of protein level (Fig. 8B). Figure 8C shows that RAD51 localized at the sites of DNA damage interacted with nuclear CHK1. These data indicate that nuclear CHK1 works on the HR repair as the degree of RAD51. In SKBR-3, decreased nuclear CHK1 by the ATR inhibition impairs

DNA repair and leads to the accumulation of DNA damage due to HR inactivation. BT-474, on the other hand, could repair the DNA damage owing to intact nuclear CHK1 but there is no response to chemotherapeutic drug. The decreased phosphorylation (S345) of CHK1 which was observed through nuclear CHK1 expression influences upon the sensitivity of ATR inhibitor.

(A) CHK1 localization

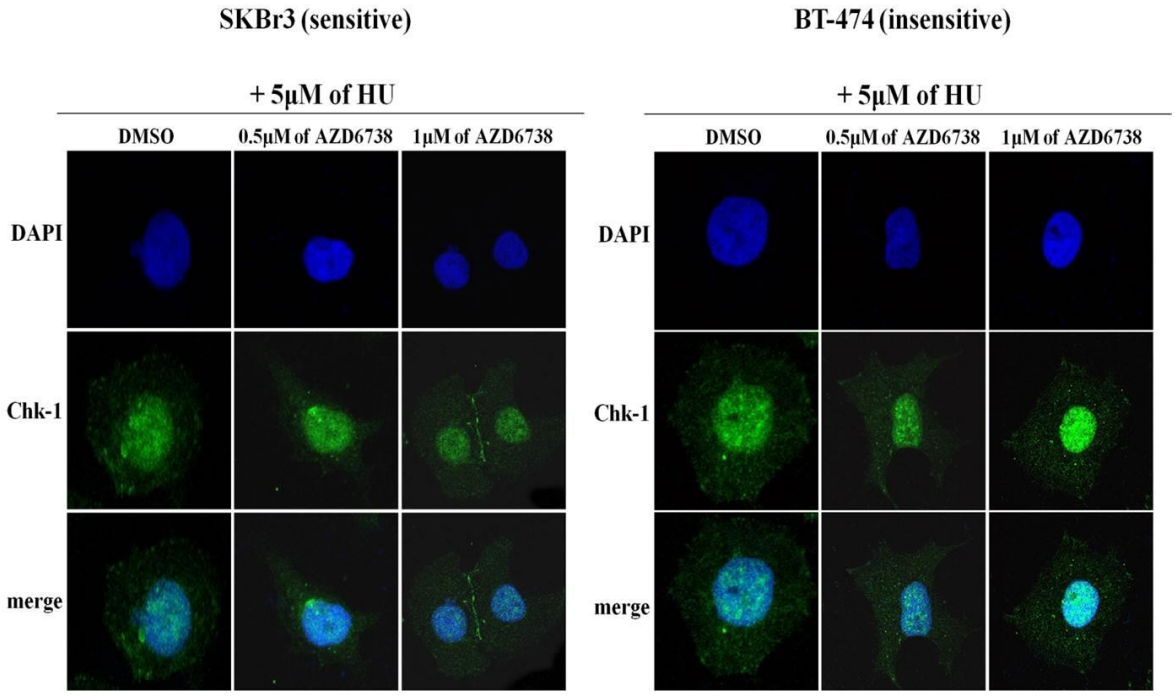
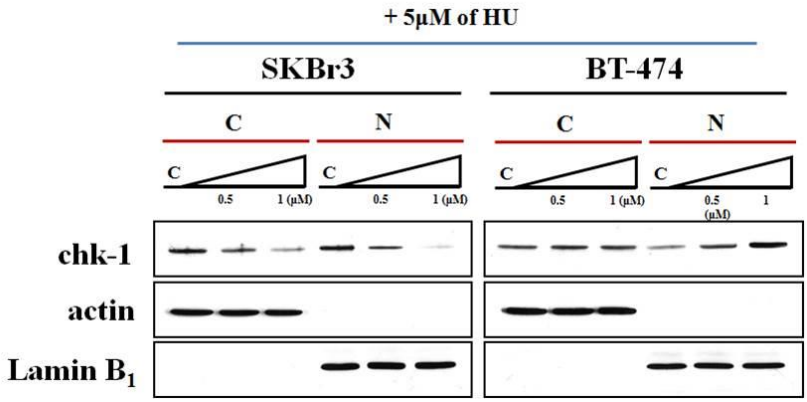


Figure 8. The levels of nuclear CHK1 expression determines the efficiency of HR DSB repair

(B) Nuclear CHK1 expression



(C) Nuclear CHK1 interaction

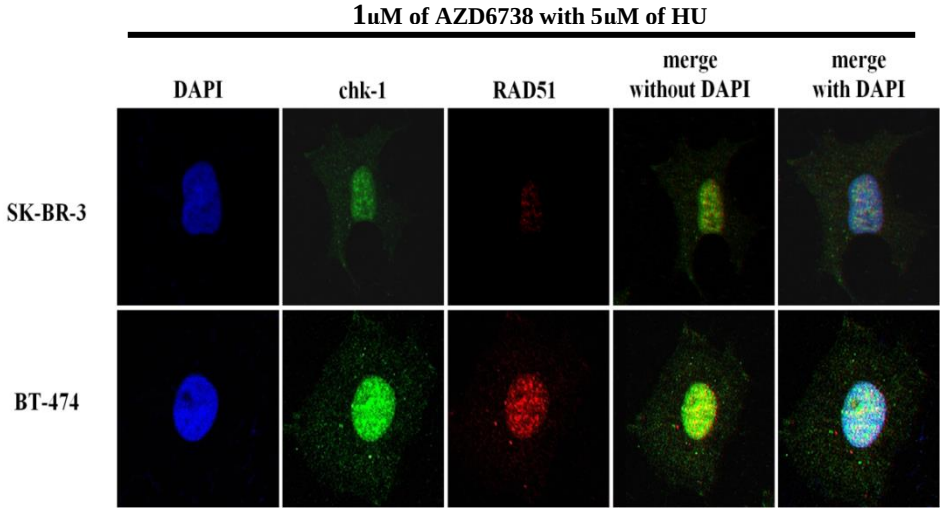


Figure 8. The levels of nuclear CHK1 expression determines the efficiency of HR DSB repair

AZD6738 with chemotherapeutic agents results in additive or synergistic inhibitory effect

Since previous research also demonstrated that ATR inhibition enhances cellular sensitivity to chemo- and radiotherapy (36), we hypothesized that co-treatment of AZD6738 with DNA damaging agent, cisplatin and microtubule inhibitor, paclitaxel would be effective in breast cancer cell lines.

To characterize the level of the interaction (synergistic, additive or antagonistic) between AZD6738 and cisplatin or paclitaxel, combination index (CI) values were calculated based on the Chou and Talalay median-effect principle. A CI is 1 for additive interaction, greater than 1 for antagonistic interactions, and less than 1 for synergistic interaction.

In the breast cancer cell lines, synergistic effect of the combinational interaction of AZD6738 with cisplatin or paclitaxel was shown regardless of subtype (Table 3 and 4). These data suggest that AZD6738 sensitized cancer cells to DNA damaging agent and microtubule inhibitor.

Table 3. Combination of AZD6738 and cisplatin shows synergism in some breast cancer cells.

Combination effects on AZD6738 and cisplatin in breast cancer cell lines				
Cell lines	**AZD6738 IC_{50} (μmol/L, mean ± SD)**	**cisplatin IC_{50} (μmol/L, mean ± SD)**	**1:10 combination IC_{50} (μmol/L, means ± SD)**	**Combination index (ED75)[†]**
BT-549 (TNBC)	0.62±0.12	1.35 ± 0.01	0.14 ± 0.004	0.13957
HCC70 (TNBC)	0.61±0.028	1.35 ± 0.005	0.107 ± 0.002	0.1553
MDA-MB-468 (TNBC)	0.98±0.03	0.56 ± 0.009	0.029 ± 0.0005	0.3362
MDA-MB-231 (TNBC)	0.74±0.13	6.41 ± 0.2	0.26 ± 0.01	0.66774
MDA-MB-157 (TNBC)	0.91±0.2	5.04 ± 0.31	0.94 ± 0.002	1.06466
HCC1143 (TNBC)	0.90±0.09	1.04 ± 0.02	0.78 ± 0.011	1.07473
Hs578T (TNBC)	>1	4.81 ± 0.4	3.11 ± 0.011	13.8326
MDA-MB-453 (HER2)	0.47±0.17	2.34 ± 0.002	0.12 ± 0.004	0.07563
SK-BR-3 (HER2)	0.59±0.08	1.27 ± 0.002	0.11 ± 0.004	0.32628
BT474 (HER2)	>1	>10	>1	6.55E+07
MCF-7	0.64±0.07	2.34 ± 0.0003	0.17 ± 0.003	0.46224
T-47D	>1	1.78 ± 0.02	0.97 ± 0.011	0.65004

Table 4. Combination of AZD6738 and paclitaxel shows synergism in some breast cancer cells

Combination effects on AZD6738 and paclitaxel in breast cancer cell lines.

Cell lines	AZD6738 IC_{50} (μmol/L, mean ± SD)	paclitaxel IC_{50} (μmol/L, mean ± SD)	1:10 combination IC_{50} (μmol/L, means ± SD)	Combination index (ED75)
HCC70 (TNBC)	0.61±0.028	0.017 ± 0.00008	0.006 ± 0.00006	0.01463
MDA-MB-468 (TNBC)	0.98±0.03	0.002 ± 0.0001	0.002 ± 0.00002	0.19855
MDA-MB-157 (TNBC)	0.91±0.2	0.008 ± 0.0002	0.005 ± 0.0003	0.2262
MDA-MB-231 (TNBC)	0.74±0.13	0.007 ± 0.0002	0.007 ± 0.0001	0.27929
BT-549 (TNBC)	0.62±0.12	0.011 ± 0.0004	0.004 ± 0.000004	0.29309
HCC1143 (TNBC)	0.90±0.09	0.005 ± 0.00002	0.005 ± 0.00001	0.37972
Hs578T (TNBC)	>1	0.007 ± 0.00006	0.008 ± 0.0007	7.848
SK-BR-3 (HER2)	0.59±0.08	>1	0.004 ± 0.0002	0.27938
BT474(HER2)	>1	>1	>1	13.313
MDA-MB-453 (HER2)	0.47±0.17	>1	>1	2.2725
MCF-7	0.64±0.07	>1	0.005 ± 0.0001	0.12239
T-47D	>1	0.004 ± 0.0001	0.003 ± 0.0008	3.033

DISCUSSION

DNA repair mechanisms play an essential role in promoting genomic stability. Defective DNA repair may predispose to cancer. On the other hand, impaired DNA repair capacity in cancer cells may influence a favorable response to chemotherapy and radiotherapy. More recently, DNA repair has emerged as a new area for anticancer drug discovery. The most advanced class of DNA repair inhibitors to date are PARP inhibitors, which has potential as a therapeutic agent to treat cancers with BRCA1 and BRCA2 mutation (37-39). Many different kinds of DDR inhibitors are being developed. Because the checkpoint response of the so-called DDR relies on two members of the PI3K-related kinase family, ATM and ATR (40), ATR inhibitor is one of the developing DDR inhibitors. ATM is primarily activated by DNA double-strand breaks (DSBs), whereas ATR responds to a much broader spectrum of DNA damage, including DSBs and many types of DNA damage that interfere with DNA replication (19, 41). In contrast to ATM, which mainly provides a rapid protective response to an extremely lethal form of DNA damage, ATR has a crucial role in stabilizing the genomic integrity throughout cell cycles and thus is essential for cell survival (20). In addition, the substrates of ATR are known as its function in protein modification, transcriptional regulation, developmental processes, cell structure, mobility, proliferation and differentiation. ATR is known to play an essential role in cell proliferation (42, 43). This study showed

alteration of intracellular signal pathways involved in cell proliferation by treatment of ATR inhibitors with in vitro models. There have been some studies studies that ATR inhibitors in combination with chemotherapy or radiotherapy radiotherapy can increase cancer cell killing (37, 44). The greater part of them showed that the cytotoxic effects were enforced by chemotherapy or radiotherapy combined with ATR inhibitor. Despite the great potential of ATR inhibitors, few studies have demonstrated the effect of ATR inhibitor monotherapy. In the present study, ATR inhibitor monotherapy effect was firstly confirmed in breast cancer cells. ATR inhibition by AZD6738 resulted in a heterogenous response among breast cancer cells using the MTT assay. Results of MTT showed that the profound sensitization of especially among HER2 positive breast cancer cell lines to chemotherapy according to PI3K mutation. Thus, further experiments were conducted to study the difference in the mechanism of action of AZD6738 in HER2 positive cell lines. It was identified that the treatment effect of ATR inhibitor was different according to the degree of nuclear localization of CHK1. The checkpoint kinase CHK1 regulates mitotic progression in response to DNA damage and replication interference. CHK1 is phosphorylated on Ser-317 and Ser-345 following a checkpoint signal, a process that is regulated by ATR (35). The phosphorylated CHK1 on Ser-345 serves to localization/retention of CHK1 in the nucleus (35).

At the same HER2-positive subtype, ATR inhibition combined with DNA damage stimuli induced the differences of CHK1 localization and this result was supposedly correlated with AKT activation. BT-474, insensitive to ATR inhibitor, presented the cell line accompanied with PI3K mutation and AKT upregulation.

It has been reported that AKT induces upregulation of non-homologous end joining (NHEJ) pathway, which plays the role of inactivation of homologous recombination (HR) for the repair of DNA damage (37-39). At the same AKT induced KU-80/ DNA protein kinase catalytic submit (DNA-PKcs) complex formation and accumulation of DNA-PKcs to DNA damage site (39); and AKT provokes activation of CHK1 which proceeded from phosphorylation of CHK1 that is KU-80 molecule dependent (37, 45). It is regarded that the ascertainment of an AKT's influence on localization of CHK1 under the accompanied DNA damage condition by ATR inhibition in the BT-474 cell line, could help understand the correlation between oncogenic pathway and DNA damage response. In this study, localization of CHK1 was detected in two cell lines (SKBR-3 and BT-474), This result found out that the localization of CHK1 blocks HR repair which is RAD51 dependent and prevents the repair of DNA damage that could be generated during ATR inhibition and finally the repeated damage repairs were accumulated and led to cell death. In the basis of the result, this study enabled the deeper understanding of nuclear CHK1 as biological function at the DDR pathway. In conclusion, nuclear localization of CHK1 is an important determining factor of the sensitivity of ATR inhibitor.

It was identified that ATR inhibitor, AZD6738, induced G2/M arrest in triple negative breast cancer (TNBC) BT549while AZD6738 aroused S phase arrest of cell cycles in HER2 positive subtypes. This result gives a demonstration of S-phase arrest as well as G2/M phase arrest by the inhibition of ATR. Nishida et al. observed the same effect of ATR inhibition

that schisandrin B, potential ATR inhibitors inhibited not only the G2/M phase but also the S-phase checkpoints in DNA damage response induced by UV (44). Huntoon et al. demonstrate that in ovarian cells exposed to cisplatin or topotecan, the addition of ATR inhibitor abrogated the S-phase (cisplatin) and G2/M (cisplatin and topotecan) accumulations induced by these agents (46). This result presents that the inhibition function of ATR could work on S-phase arrest as well as G2/M phase arrest, and through this fact, it is expected that the antitumor effects for breast cancer cells can be fortified by AZD6738's inhibiting the function of the broader cell cycles.

In the BT-474 cell lines, phosphorylated extracellular signal-related kinase (ERK) was increased after the treatment of ATR inhibitor in a dose-dependent manner. ERK activation via MEK was observed in response to multiple DNA damage stimuli (47, 48). ERK is primarily linked with cell proliferation and survival, and seems to prevent apoptosis. ERK pathway is crucial in efficient HR repair and induced ATM activation, suggestive of a regulatory feedback loop between ERK and ATM (49). ERK induced upregulation of DNA-PKcs which is necessary for genomic stability at NHEJ as well as HR (50). Marampon et al. showed that ERK inhibition enhances radiosensitivity of rhabdomyosarcoma cells because of the decreased DDR capacity (50). By DNA damage generated during ATR inhibitor treatment in the BT-474 cell line, ERK increases to promote activation of DDR pathway. Through the series of this process, DNA repair molecules such as nuclear CHK1 and RAD 51 foci are activated and DDR can be intensified. Activated ERK eventually induced the resistance to ATR inhibitor in BT-474 cell line.

According to the previous studies, the effects of ATR inhibitor have been reported with other cancer cells. However, how ATR inhibitor plays an essential role in repairing DNA damage in breast cancer cells, and what mechanisms induced blocking of DNA damage repair have not yet been understood. This is the first report showing the effects of ATR inhibition on breast cell lines. Additionally, this study suggests that ATR inhibitor could be a sensitizer of mitotic inhibitor, paclitaxel, as well as DNA damaging agent, cisplatin in the breast cancer cell lines. It would be expected to be a new strategy for the treatment of breast cancers and for example, in case that taxane shows poor treatment effects,, cytotoxic effects could be magnified through the adding of ATR inhibitor in vitro. Furthermore, these findings provide a preclinical rationale of conducting future clinical trials evaluating the use of AZD6738 for treating breast cancer patients.

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CONCLUSIONS

ATR inhibitor, AZD6758 has anti-proliferative effects on human breast cancer cell lines. AZD6738 downregulates the signal of proliferation and induces growth arrest in the S phase and increased apoptosis in sensitive cell line, SKBR-3. The results of this study defined that ATR inhibition disrupts functional localization of CHK1 resulting in HR inactivation. Increased levels of DNA repair capacity could promote resistance to chemotherapy in breast cancer. The combination of AZD6738 with chemotherapeutic agents results in additive or synergistic inhibitory effect as a chemo-sensitizer.

Overall, these data suggest that AZD6738 could be a potential therapeutic agent not only alone but also in combination with chemoagents on human breast cancer.

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국문 초록

배경 : DNA 복구 기전은 유전체 항상성을 유지하는데 중요한 역할을 하며, DNA 손상 복구 과정에 결함이 있으면, 유전체 불안정성을 일으킨다. 암세포에서 DNA 손상 복구 기전의 활성화는 항암제 내성의 중요한 기전이 되고 있으며, DNA 손상 복구 기전의 결함은 분화도가 나쁜 암과 관련이 있다. DNA 손상 복구 기전은 DNA 의 손상을 감지하는 기능에서 시작되는데, ataxia telangiectasia and Rad3-related (ATR) 은 DNA 손상 복구 기전의 감지 및 조절에 중요하다. 그러므로 ATR을 새로운 표적으로 이용하여 종양의 성장을 억제하려는 방법은 유용할 것으로 생각되어, 본 연구에서는 유방암 세포주에서 ART 억제제 (AZD6738)의 항종양효과를 확인하고 기전을 밝히고자 하였다.

방법 : MTT 분석을 시행하여 AZD6738 의 성장억제 효과를 보았고, 세포주기 분석과 DNA 손상 복구 기전에 관여하는 단백질과 세포 신호 전달에 관여하는 단백질의 발현을 AZD6738 처리 전과 후에 비교하여 ATR 억제제가 미치는 영향을 분석하였다. 면역형광법 및 comet assay 를 이용하여 AZD6738 이 DNA 손상 복구 기전에 미치는 영향을 분석 하였다.

결과 : ATR 억제제인 AZD6738 은 유방암 세포주에서 항종양효과를 나타내었다. AZD6738 은 S phase 세포주기 억제, 세포사멸 유도, 세포증식 기전의 억제를 통해 항종양효과를 보였다. AZD6738 은 ATR 억제제에 민감한 SKBR-3 세포에서 pCHK1(S345), RAD51, MRE11 및 ERCC1 발현 감소를 유도 하였고 RAD51 foci 의 형성을 감소 시켰다. 또한 cisplatin 과 paclitaxel 과 함께 사용시 유방암에서 항암제의 효과를 증가 시켰다. ATR 억제제는 세포에서 유전자 손상을 인지하지 못하게 하고 상동 재조합의 기능저하가 유발됨에 따라서 유전자 손상이 축적되고 이는 유전적 불안정성을 야기하여 세포사멸로 이어지게 됨을 알 수 있었다.

결론 : ATR 억제제는 유방암 세포주에서 세포증식과정에 영향을 미치고 CHK1 에 의존적인 상동재조합에 관여한다. 또한, ATR 억제제는 항암제의 효과를 증가 시키는 sensitizer로도 작용한다. 그러므로 향후 ATR 억제제 단독 혹은 다른 항암제와의 복합치료를 이용한 임상시험의 기반을 마련하였다.

주요어: DNA 손상 복구 반응 ATR 억제제, 유방암, 상동 재조합

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