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# 인체 위암세포주에서 pan-Pim 억제제인 AZD1208의 항종양효과 및 Akt 억제제와의 상승 효과에 관한 연구

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협동과정 종양생물학 전공

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# Pan-Pim kinase inhibitor AZD1208 suppresses tumor growth and synergistically interacts with an Akt inhibitor in gastric cancer cells

By

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

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# ABSTRACT

## Pan-Pim kinase inhibitor AZD1208 suppresses tumor growth and synergistically interacts with an Akt inhibitor in gastric cancer cells

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Pim kinases are highly conserved serine/threonine kinases that control proliferation and survival pathways in cancer cells. Different expression patterns of each Pim isoform (Pim-1, Pim-2 and Pim-3) have been observed in various types of human cancer including gastric cancer. AZD1208 is a potent and selective inhibitor that affects all three Pim kinase isoforms. In the present study, we used AZD1208 to

demonstrate that inhibition of Pim kinase activity induces gastric cancer cell death. Treatment with AZD1208 alone down-regulated the expression of the Pim kinase substrates, but cell viability in short-term cell cultures was not observed. In long-term cultures, we observed significantly decreased cell viability and up-regulated expression of light chain 3B (LC3B), an autophagy marker, in a dose-dependent manner only in the AZD1208-sensitive cell line. This finding suggested that the cytotoxic effect of AZD1208 was achieved through autophagy and not apoptosis. Moreover, we found that ataxia telangiectasia mutated (ATM) and checkpoint kinase (CHK2), two critical components of the DNA repair pathway, were activated in an AZD1208-resistant cell line. We also confirmed that a combination of an Akt inhibitor with AZD1208 produced a highly synergistic effect in gastric cancer cell lines. Taken together, our data demonstrated that treatment with AZD1208 alone induced considerable cell death through autophagy. Additionally, a combination of Pim and Akt inhibitors had a strongly synergistic effect in human gastric cancer cell lines.

**Keyword: AZD1208, Pim kinase inhibitor, autophagy, cell death, Akt, gastric cancer**

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# INTRODUCTION

Pim kinases are a class of constitutively active serine/threonine kinases consisting of three highly homologous members: Pim-1, Pim-2, and Pim-3. These factors were discovered as proviral insertion sites of the Moloney murine leukaemia virus associated with the development of T-cell lymphomas [1]. Members of this kinase family have been implicated in several biological process including cell survival, proliferation, and differentiation [2]. Pim kinase expression is mainly regulated by transcription factors such as Janus kinase/Signal transducer and activator of transcription, JAK/STAT, the nuclear factor kappa-B, NF-κB, pathway, and ubiquitination with subsequent proteosomal degradation at the post-translational level [3].

The Pim kinases modulate cell proliferation by directly binding to cell cycle regulators such as p21, p27, Cdc25A, and Cdc25C, and mediate survival signal pathways by phosphorylating the Bcl-2 antagonist of cell death (Bad) [4, 5]. Furthermore, these kinases have been found to induce the phosphorylation of 4E-binding protein 1(4E-BP1), which permits protein synthesis by 5'cap-dependent translation [4, 6, 7].

When the Pim kinases become hyperactivated or uncontrolled by oncogenic signal, genetic alteration or DNA damage, they represent

several hallmarks of cancer [2]. Mechanistic studies have revealed that high expression levels of Pim are associated with tumorigenesis. For example Pim-1 and Pim-2 are commonly overexpressed in case of hematologic disease and some solid tumors [2, 4]. In particular, there is a relationship between aberrant expression of Pim-3 and gastric cancer progression [8]. Pim expression is also correlated with poor prognosis and therapy outcomes [9-11], Thus, Pim kinases have become interesting targets for new drug development. Previous research has shown that a small molecule inhibitor of Pim kinases lead to considerable cell death in case of haematological cancer [12-15]. However, this has not been reported in gastric cancer.

Dysregulation of the phosphatidylinositide 3-kinases, PI3K/Akt pathway is commonly found in many types of human cancer including gastric cancer [16, 17]. Therefore, inhibition of this pathway is an attractive therapeutic target for treating gastric cancer. Although small molecular inhibitors that target Akt have been developed, these compounds were associated several problems including poor pharmacokinetics, relatively weak inhibition, toxicity, and activation of compensatory signalling pathways [18-20]. Notably, previous studies showed that Pim and Akt kinases had partially overlapping substrates, but independent signal pathway. Furthermore, up-regulation of receptor

tyrosine kinases by Pim could induce resistance of Akt inhibitor [17, 21]. For this reason, simultaneous inhibition of Pim and the Akt pathway has beneficial anticancer therapeutic effects.

In present study, the antitumor activity of a Pim inhibitor, AZD1208, was evaluated in a panel of gastric cancer cell lines including SNU series which was established from our patients and maintained through Korean Cell Line Bank. The underlying mechanism was also examined. Subsequently, the role of AZD1208 as a sensitizer when administered in combination with an Akt inhibitor was assessed. This is the first investigation to show that the treatment with AZD1208 alone exerts a cytotoxic effect by inducing autophagy. Furthermore, dual inhibition of the Pim and Akt pathways cooperatively decreased cell viability and improved drug efficacy in human gastric cancer cells.

# PURPOSE OF THIS STUDY

**1)** To investigate anti-tumor effect of AZD1208 in human gastric cancer cell lines.

**2)** To understand the molecular action mechanism of AZD1208.

**3)** To evaluate the efficacy of AZD1208 in combination with Akt inhibitor in gastric cancer cell lines.

# MATRIALS AND METHODS

### *1. Reagents*

AZD1208, a Pim inhibitor [22], and AZD5363, an Akt inhibitor were kindly provided by AstraZeneca (Macclesfield, Cheshire, UK). The compound was initially dissolved in dimethyl sulphoxide (DMSO) at a concentration of 3.79mg/mL. Aliquots of the solution were then stored at -80℃. 3-methyladenine (3-MA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-fmk) was obtained from R&D System (Minneapolis, MN, USA).

### *2. Cell lines and cell culture*

Human gastric cancer cell lines (SNU-1, -5, -16, 216, -484, -601, -620, -638, -668, -719, AGS, MKN45, KATO-III, and N87) were purchased from the Korean Cell Line Bank (Seoul, South Korea). Identities of the cell lines were authenticated by DNA fingerprinting analysis [23]. All cell lines were banked and passaged for less than 6 months before use. The cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Welgene, Deagu, Korea) and 10μg/mL gentamicin(Cellgro, Manassas, VA, USA) at 37℃ in 5% $CO_2$.

### *3. Cell growth inhibition assay*

Cells (2-3 x $10^3$ in 100μL/well) were seeded in 96well plates (TPP, Trasadingen, Switzerland) and incubated overnight at 37℃ in 5% $CO_2$. The cells were then exposed to increasing concentrations of Pim inhibitor (ranging from 0 to -10μmol/L) for 5 d. After 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 VersaMax™ microplate reader (Molecular Devices; Sunnyvale, CA, USA). The absorbance and $IC_{50}$ of the Pim inhibitor were analyzed using SigmaPlot software [Statistical Package for the Social Science, Inc. (SPSS), Chicago, IL, USA]. Six replicate wells were included for each analysis and at least three independent experiments were conducted.

### *4. Colony formation assay (CFA)*

For the CFA, cells were seeded into 6-well plates (TPP, Trasadingen, Switzerland) and incubated for 48 h at 37℃ in 5% $CO_2$. Next, the cells were treated with various concentrations (0.5, 1, and 5 μmol/L) of AZD1208 alone or in combination with 500nmol/L AZD5363 every 3

days. The cells were cultured until colonies formed (14 days). After removing the culturing media, the cells were washed in phosphate-buffered saline (PBS) and stained with a 0.1% Coomassie Blue solution (Sigma Aldrich) for 1 hour at room temperature. The excess staining solution was then removed, and the plates were washed in PBS and air-dried. The cell colonies were counted using a GelCountTM automatic plate scanner (Oxford Optronics GelCount, Oxford, UK). The cell survival rate and $IC_{50}$ of AZD1208 were determined using SigmaPlot software (SPSS).

5. ***Reverse transcription(RT)-PCR and real-time PCR***

Total RNA was isolated using TRI reagent®(Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA was synthesized by performing RT-PCR with ImProm-II™ reverse transcriptase (Promega; Madison, WI, USA) and amplified using AmpliTaq Gold® DNA polymerase (Applied Biosystems; Carlsbad, CA, USA) with gene-specific primers. Quantitative real-time(qRT)-PCR was conducted using an iCycler iQ detection system (Bio-Rad Laboratories, Inc.; Hercules, CA, USA) with SYBR Green.

Data from all samples were normalized relative to actin cDNA. Sequences of the primers used for RT-PCR and qRT-PCR are listed in Table 1. cDNA was synthesized at least three times from three

independent sets of samples, and all PCR analyses were conducted in triplicate.

**Table 1. Sequences of the primers for PCR and real-time PCR analyses.**

| Gene | Primer sequence (5'→3') | Size (bp) |
|---|---|---|
| Pim-1 | F: GGATCCGCTACCATCGCTAC<br>R: GGCAGGAGAACATCTTGCAT | 253 |
| Pim-2 | F: GTGGCCATCAAAGTGATTCC<br>R: CTGGATGGCTGCCACTACTT | 285 |
| Pim-3 | F: GAAAATCTGCTTGTGGACCTG<br>R: CACTCTGGAGAGACCCTCCTC | 278 |
| Actin | F: AGAGCTACGAGCTGCCTGAC<br>R: GGATGCCACAGGACTCCA | 206 |

### *6. Western blot analysis*

Cells were collected after drug treatment, washed with ice-cold PBS, and incubated in extraction buffer [50mM Tris-Cl (Ph7.4), 150mM NaCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM sodium fluoride, 1mM sodium pyrophosphate, 2mM phenylmethylsulfonyl fluoride, 1mg/mL pepstatin A, 0.2mM leupeptin, 10μg/Ml aprotinin, 1 mM sodium vanadate, 1mM mitrophenylphosphate, 5mM benzamidine, and 500 μM cantharidine] 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 a 7%-15% SDS-polyacrylamide gel. The separated proteins were transferred onto

nitrocellulose membranes, and the blots were probed with primary antibodies (1:1000 dilution) overnight at 4℃.

The antibodies used were as follows. Pim-1 (sc-13513, Santa Cruz Biology Technology, CA, USA), Pim-2 (CST 4730, Cell Signaling Technology, Beverly, MA), Pim-3 (CST 4165), pBad serine 112 (CST 9296), Bad (CST 9292), p4EBP1 S65 (CST 9451), 4EBP1 (CST 9452), pS6K (CST 2211), S6K (CST 2217), LC3B (CST 3868), Beclin-1 (ab51031), pATM serine 1981 (CST 4526), ATM (sc-23921), pChk2 threonine 68 (CST 2661), Chk2 (sc-5278), α-tubulin (Sigma Aldrich). Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer's protocol (Amersham Biosciences; Piscataway, NJ, USA).

### *7. Cell-cycle analysis*

Cells treated with AZD1208 and/or AZD5363 were harvested, fixed with cold 70% ethanol, and then stored at -20℃ for at least 24 h. Next, the cells were washed in PBS, incubated with 10 μg/mL RNase A (Sigma-Aldrich) at 37℃ for 20 min, and stained with 20 μg/mL propidium iodide (PI; Sigma-Aldrich). DNA contents of the cells (10,000 cells per group) were quantified using a FACS Calibur flow cytometer (BD Biosciences; San Jose, CA, USA ).

### *8. Plasmid and siRNA transfection*

The LC3 fusion plasmids, pCMV-GFP-LC3, was purchased from Cell Biolabs (San Diego, CA, USA). siRNAs specific for Pim was obtained from Genolution Pharmaceuticals (Seoul, Korea). SNU-638 cells transfected with siRNA at the indicated concentration or 1μg of LC3 plasmid using G-fectine (Genolution) and Mirus transfection agent (Mirus Bio, Madison, WI) respectively according to the manufacturer's instructions. After 72 h, the cells were harvested and subjected to western blotting. The sequence of the Pim-1-specific siRNA was 5'-ACCAUCCAUGGAUGCAAGAUG UUCUUU-3', the sequence of the Pim-2-specific siRNA was 5'-GUGGAGUUGUCCAUCGUGACAUU -3', and the sequence of the Pim-3-specific siRNA was 5'-GGCGUGC UUCUCUACGAUAUU-3'. The sequence of the NT siRNA was 5'-AA TTCTCCGAACGTGTCACG-3'.

### *9. Immunofluorescence assay (IFA)*

Cells were plated on 0.01% poly-L-Lysine (Sigma Aldrich)-coated coverslips, transfected with GFP fluorescent LC3B and treated with 1 or 5μM of AZD1208. After 5 d, the coverslips were rinsed once in PBS (37℃), fixed in 3.7% paraformaldehyde for 10 min, permeabilized with PBS-T(0.5% Triton-100 in PBS) for 5 min, and incubate with primary

antibody for 24 hours at 4℃. The primary antibodies used in this study were rabbit polyclonal anti-LC3B 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 (300nM; Invitrogen) and the coverslips were mounted onto slides using Faramount aqueous mounting medium (Dako; Denmark). Immunofluorescence was visualized using a Nikon A1 confocal laser scanning microscope (Nikon, Tokyo, Japan).

### *10. 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.

# RESULT

## 1. AZD1208 inhibits the growth of gastric cancer cells.

To determine the effects of AZD1208 on human gastric cancer cell growth, we first treated each cell line with various concentration of AZD1208 for 120 h. Cell survival was then measured with an MTT assay (Figure 1). AZD1208 had a minimal cytotoxic effect on most of the cell lines up to a concentration of 1 μM. Cell survival rates range between 77% and 98%. At 10 μM, AZD1208 had a cytotoxic effect on N87 and MKN45 cells with a cell death rate of approximately 40%. Overall, no obvious growth inhibition was observed during the early time point of the cell viability assay. Since Pim kinases play a role in diverse level, this study confirmed the efficacy in long term culture system, used CFA and observed that AZD1208 affects cell proliferation in gastric cancer cell lines. Several cell lines (i.e., SNU-484, -638, and -719) were sensitive to AZD1208 compared to other cell lines in a dose-dependent manner (Table 2, Figure 2). SNU-638 cells were highly sensitive to AZD1208 compared to other cell lines, especially SNU-601 cells that were the most resistant. Based on the CFA results, SNU-638 as a sensitive cell line and SNU-601 as a resistant cell line were chosen for further study.

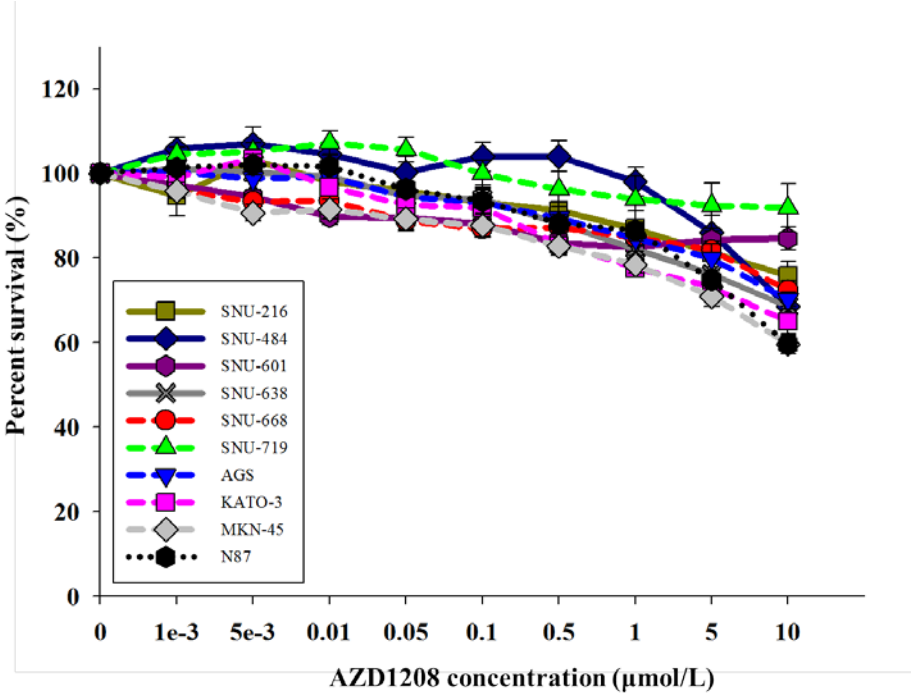



**Figure 1. AZD1208 showed minimal cytotoxic effect in gastric cancer cell lines.** Cells were incubated with different concentrations of AZD1208 for 120 h and an MTT assay was performed. Cell survival was calculated as a percentage and presented as a graph with standard error bars (n = 3)

**Table.2 Effects of AZD1208 on the inhibition of human gastric cancer cell growth.**

| Cell line | CFA : $IC_{50}$ (μmol/L, means±SD) |
|---|---|
| SNU-216 | >5 |
| SNU-484 | 3.36±0.466 |
| SNU-601 | >5 |
| SNU-638 | 1.47±0.082 |
| SNU-668 | 3.10 |
| SNU-719 | 3.11±1.47 |
| AGS | >5 |
| MKN-45 | 4.69 |
| N87 | >5 |

The growth inhibitory activity of AZD1208 was measured with a colony formation assay. Cells were seeded and treated with various concentration (0.1, 0.5, 1, 2.5 and 5 μM) of AZD1208 every 3 d. The cells were cultured for 14 d until colonies formed and stained. $IC^{50}$ values were calculated and are represented in the table.

**A**

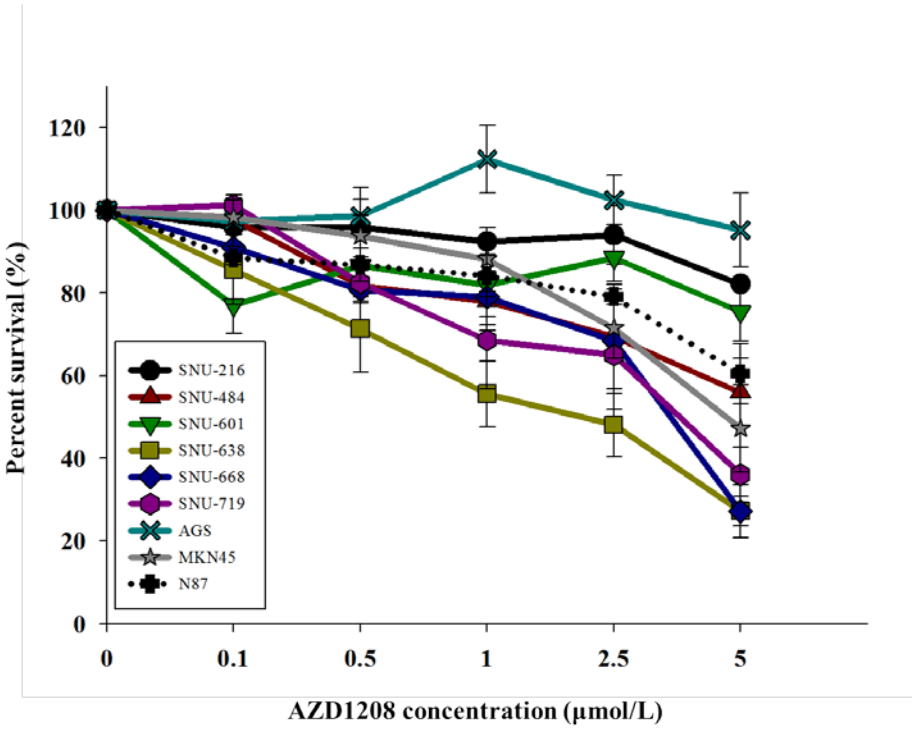



**Figure 2. AZD1208 induces growth inhibition in long term culture system. *A*,** The growth inhibitory effect of AZD1208 was evaluated by a colony formation assay. The percentage of survival cells were calculated by counting the number of colonies and presented as a graph with standard error bars (n=3).

**B**

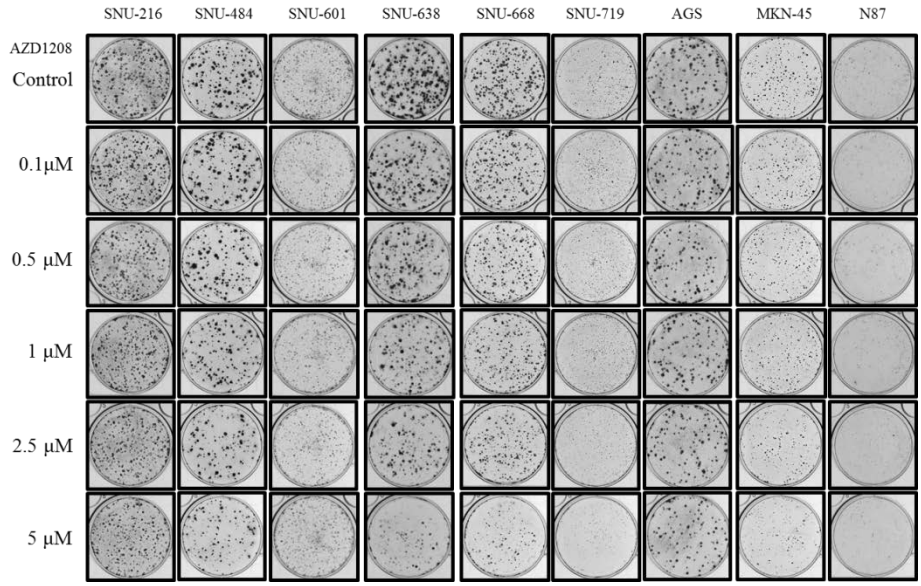



**Figure 2.** ***B,*** AZD1208 suppressed colony formation in gastric cancer cells. The cells were treated with various concentrations of AZD1208 every 3 d. The resulting colonies were stained and counted.

## 2. Alteration of PI3K pathway is associated with resistance to AZD1208

To find the predictive marker, we analyzed the mRNA and protein expression of Pim-1, Pim-2, and Pim-3 in a panel of 10 gastric cancer cell lines (Figure 3). To assess the mRNA expression, we performed qRT-PCR and RT-PCR. Differential expression of the Pim family members was seen, but mostly Pim-3 expression was up-regulation as previously reported by other groups [8]. Additionally, we observed the heterogeneous protein expression of Pim isoforms in each cell line. These data suggest that basal levels of Pim-1, Pim-2, and Pim-3 mRNA and protein did not correlate with responses to AZD1208.

However, interestingly, we found that SNU-601 and AGS harboring PIK3CA mutation showed insensitivity to AZD1208 (Table 3). It suggests that since Pim and PI3K pathway occur independently, overactivated PI3K/Akt pathway by PIK3CA mutation can induce resistance to AZD1208.

**A**

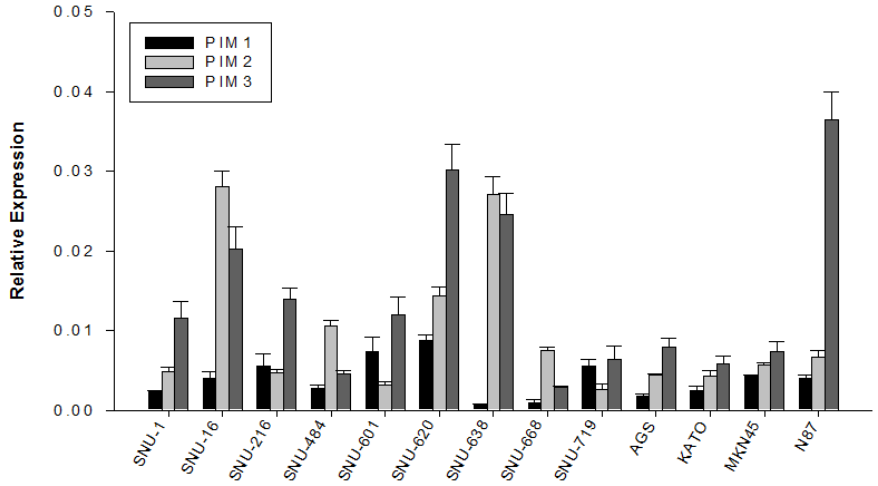



**Figure 3. Different expression levels of Pim kinases in gastric cancer cell lines.** ***A*****,** Basal mRNA expression levels of Pim kinases in human gastric cancer cell lines. Quantitative real-time reverse transcription-PCR analysis of Pim-1, Pim-2, and Pim-3 expression in human gastric cancer cell lines. Data were normalized relative to actin. The expression levels were calculated as percentages and presented as a graph with standard error bars (n = 3).

**B**

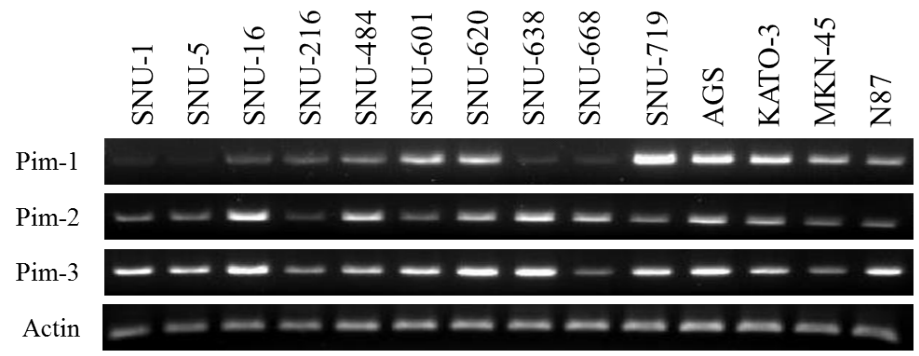



**Figure 3.** ***B,*** The expressioin levels of Pim-1, -2, and -3 were measured by RT-PCR. Total RNA was isolated from gastric cancer cells and the indicated genes were amplified with specific primers.

**C**

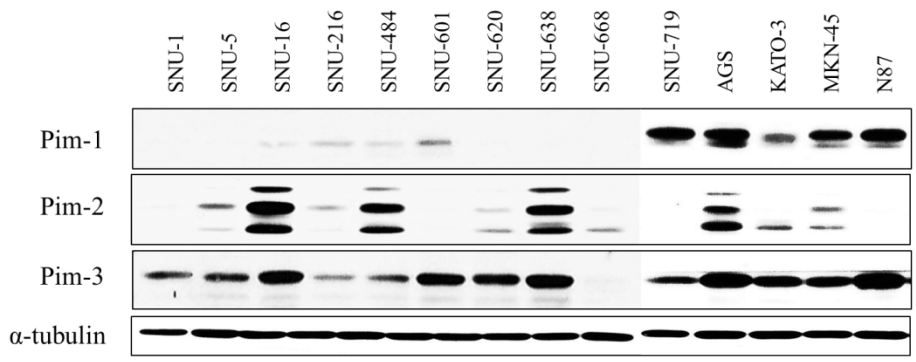



**Figure 3.** ***C,*** Basal levels of Pim kinase proteins in the human gastric cancer cell lines were confirmed by Western blot.

**Table 3. Genetic background of gastric cancer cell lines**

| Gastric Ca. | HER2 | PIK3CA | TP53 | KRAS | $IC_{50}$ (μmol/L, mean±SD) |
|---|---|---|---|---|---|
| SNU-216 | Amp. | wt | mt | wt | >5 |
| SNU-484 | Normal | wt | mt | wt | 3.36±0.466 |
| SNU-601 | Normal | E542K | mt | mt | >5 |
| SNU-638 | Normal | wt | mt | wt | 1.47±0.082 |
| SNU-668 | Normal | wt | mt | mt | 3.10 |
| SNU-719 | Normal | wt | wt | wt | 3.11±1.47 |
| AGS | Normal | E453K | mt | wt | >5 |
| MKN-45 | Normal | wt | wt | wt | 4.69 |
| N87 | Amp. | wt | wt | wt | >5 |

Amp, amplification; wt, wild type; mt, mutation

### 3. AZD1208 inhibits the phosphorylation of Pim kinase substrates.

Next, we wanted to elucidate the mechanism of AZD1208 action in sensitive and resistant cell lines. To determine whether AZD1208 was capable of inhibiting specific substrates of Pim kinase, we measured the phosphorylation and total expression levels of these target molecules by Western blotting [1, 4]. A significant dose-dependent reduction of the Pim substrates Bad and 4E-BP1 phosphorylation in SNU-638 was observed (Figure 4). In addition, phosphorylation of S6K, another downstream effector of mammalian target of rapamycin complex 1(mTORC1), was also markedly down-regulated. Because AZD1208 exerts an inhibitory effect on Pim kinase activity, phosphorylation of the Pim substrates was reduced in both cell lines but this effect was much less noticeable in the resistant SNU-601 cells. Although AZD1208 sufficiently inhibited Pim kinase activity, we could not observe any changes in cell cycle progression in either cell line at any concentration (Figure 5). Furthermore, major factors of the oncogenic proliferation pathway such as Akt and Erk were not affected by AZD1208 treatment (data not shown). Collectively, these data suggested that downstream molecules can be regulated by AZD1208 but apoptosis induction or inhibition of proliferative signaling is not

responsible for the cytotoxic effect of AZD1208.

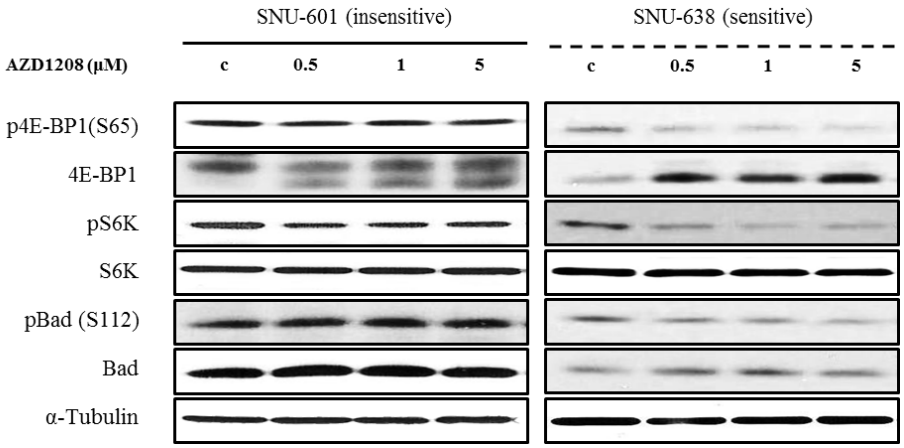
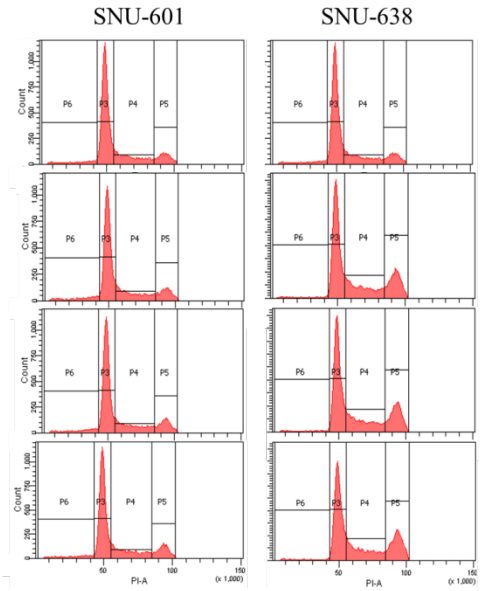



**Figure 4. AZD1208 affects downstream molecules of Pim kinase.** Cells were treated with control (DMSO) or 0.5, 1 or 5 μM AZD1208 for 1h. Western blot analysis showed that AZD1208 reduce the phosphorylation of the Pim kinase substrate proteins Bad, S6K and 4E-BP1.




| AZD1208 (120h) | Cell Cycle Phase | | |
|---|---|---|---|
| | G1 | S | G2/M |
| SNU-601 | | | |
| Control | 65.95±1.45 | 21.3±0.8 | 12.7±0.7 |
| 0.5μM | 65.75±0.85 | 21.8 | 12.35±0.75 |
| 1μM | 67.05±2.05 | 20.6±0.2 | 12.35±1.85 |
| 5μM | 65.3±2.2 | 20.95±1.35 | 13.7±0.8 |
| SNU-638 | | | |
| Control | 56.05±3.15 | 24.45±1.15 | 19.5±2 |
| 0.5μM | 55.45±2.75 | 24±0.8 | 20.6±2 |
| 1μM | 55.75±3.45 | 23.35±1.25 | 20.85±2.25 |
| 5μM | 54.05±4.05 | 24.05±1.25 | 22±2.8 |

**Figure 5. Apoptosis is not associated with cytotoxic effect of AZD1208.** Cells were treated with the indicated concentrations of AZD1208 for 5d. The percentages of cells in the G1, S and G2/M phase were determined by FACS analysis.

**4. AZD1208 induces autophagic cell death of gastric cancer cells.**

We determined whether autophagy (or type II programmed cell death) is related to the cytotoxic effects of AZD1208 because we found AZD1208 did not induce apoptosis,. First, we measured the expression levels of the well-known autophagy markers LC3B and Beclin-1 following AZD1208 treatment (Figure 6A). LC3B is cleaved to form LC3B-II during autophagy. Thus, conversion of LC3B-I to LC3B-II associated with autophagosome formation. Interestingly, AZD1208 treatment induced up-regulation in LC3B-II expression at both time points in the SNU-638 cells but not in SNU-601 cells. In contrast, Beclin-1 expression was slightly up-regulated at 36 h in SNU-638 cells, but no visible changes were found after 5 d of exposure.

Next, we performed an immunofluorescence study to further confirm the role of AZD1208 on the induction of autophagy. Both SNU-601 and SNU-638 cells were transfected with a plasmid encoding GFP-tagged LC3B. Fluorescent microscopy was then performed (Figure 6B). SNU-638 cells clearly showed a fluorescent punctate pattern indicative of LC3B expression with a concentration of 1 μM AZD1208. No significant LC3B-associated punctate pattern was observed in the SNU-601 cells.

To determine whether AZD1208 sensitivity was a direct result of

autophagy and not apoptosis, we conducted a CFA to measure the viability of SNU-638 cells in the presence of autophagy inhibitor 3-MA and caspase inhibitor z-VAD-fmk (Figure 6C). 3-MA blocked cell death in contrast to treatment with z-VAD-fmk.

In addition, we knocked down endogenous Pim isoforms with siRNA in SNU-638 cells. The cells were transfected with NT or Pim specific siRNA oligonucleotides. Since there is the possibility of compensatory functions among Pim isoforms, Pim-1, Pim-2, and Pim-3 specific siRNAs were used simultaneously. All Pim siRNAs knocked down their targets efficiently, and the triple knockdown was as efficient as the individual siRNAs separately (Figure 6D). Consistent with the above data, western blotting analysis showed triple knockdown of Pim reduced 4E-BP1 phosphorylation and increased LC3B protein levels.

Taken together, our data demonstrated that autophagic cell death induced by AZD1208 was the result of Pim modulation.

**A**

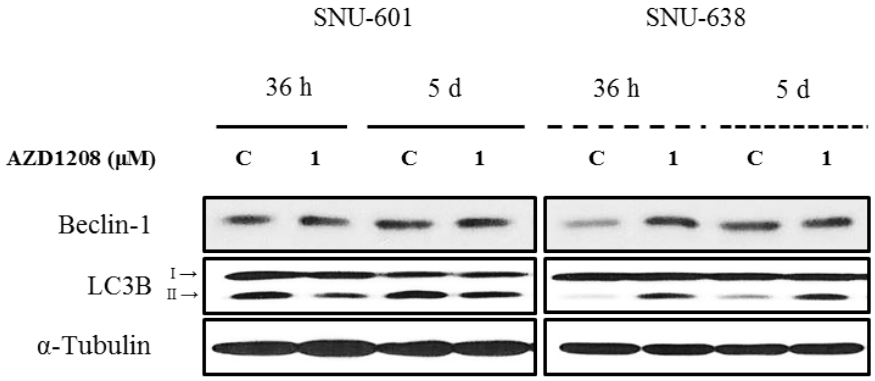



**Figure 6. AZD1208 induces cell death through stimulate autophagy**

***A,*** Cells were treated with DMSO (control) or 1 μM AZD1208 for 36 or 120 h. Expression levels of LC3B and Beclin-1 were measured by Western blot analysis.

**B**

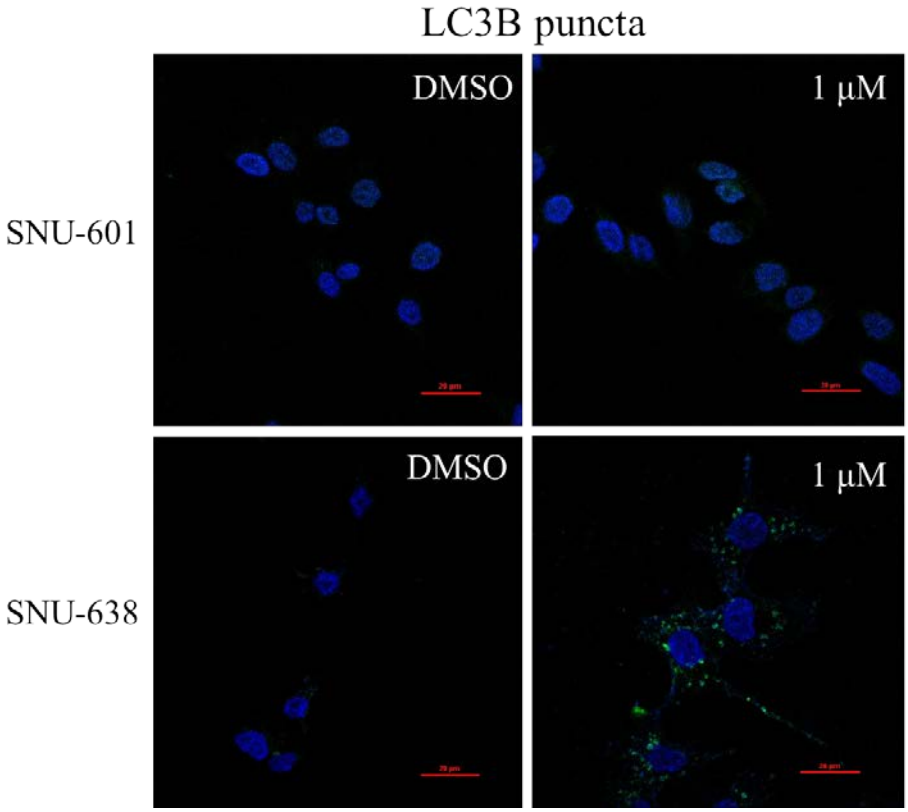



**Figure 6.** ***B,*** SNU-601 and SNU-638 cells transfected with GFP-LC3B were treated with 1 μM AZD1208. Confocal microscopy was used to observe the signals corresponding to LC3B expression (green fluorescence). DNA was counterstained with DAPI (blue). The merged picture represents overlapping signals of the two channels.

**C**

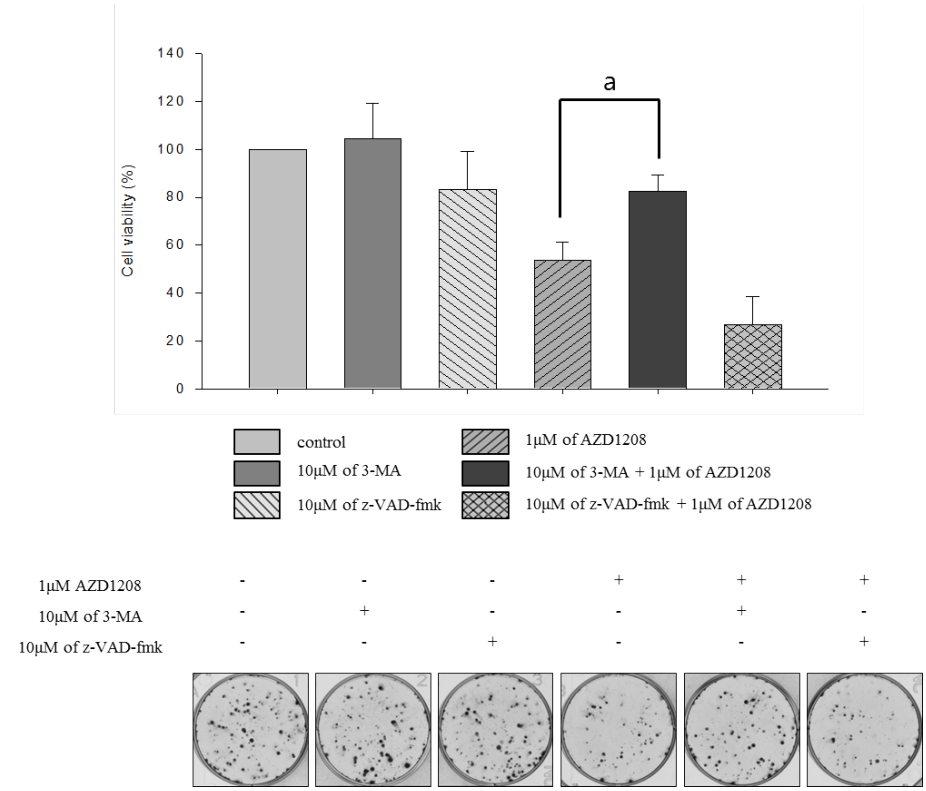



**Figure 6.** ***C,*** SNU-638 cells were pre-treated with the autophagy inhibitor 3-MA (10 μM) or caspase-3 inhibitor z-VAD-fmk (10 μM) for 24 h. Next, the cells were treated with 1 μM AZD1208 every 3 d for 14 d. The percentage of surviving cells was calculated by counting the number of colonies and presented in a bar graph with standard error bars (n = 3). a, p = 0.0082

**D**

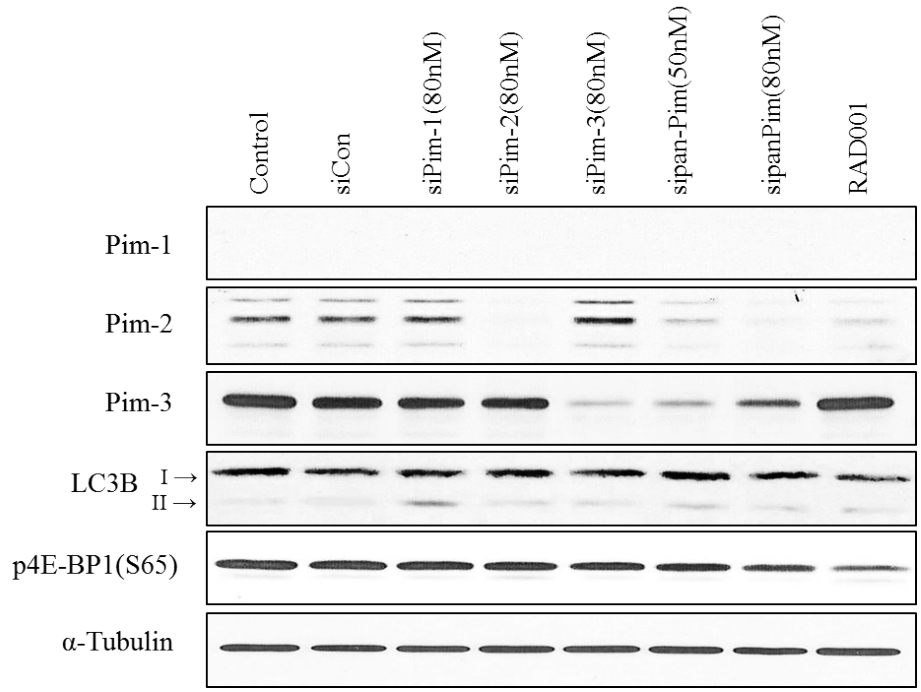



**Figure 6.** ***D,*** SNU-638 cells were transfected with Pim-1, Pim-2, Pim-3, pan-Pim or Control siRNAs for 72 h. The cells were harvested and western blotting was conducted with the indicated antibodies. 500nM of mTOR inhibitor, RAD001, was used as a positive control of autophagy.

## 5. Regulation of the DNA damage response is associated with AZD1208 sensitivity.

The roles of Pim kinases in DNA damage protection have been studied [24, 25]. We therefore determined whether AZD1208 can affect the DNA damage repair (DDR) pathway by performing a Western blot analysis (Figure 7). Intriguingly, ATM phosphorylation was up-regulated in a dose-dependent manner in SNU-601 cells with Chk2 phosphorylation. Depletion of Pim kianses can lead to DNA damage accumulation [25, 26], and regulation of the DDR may be related to drug sensitivity [27]. Our result suggests that increased activity of the DNA damage repair system could be a mechanism underlying AZD1208 resistance.

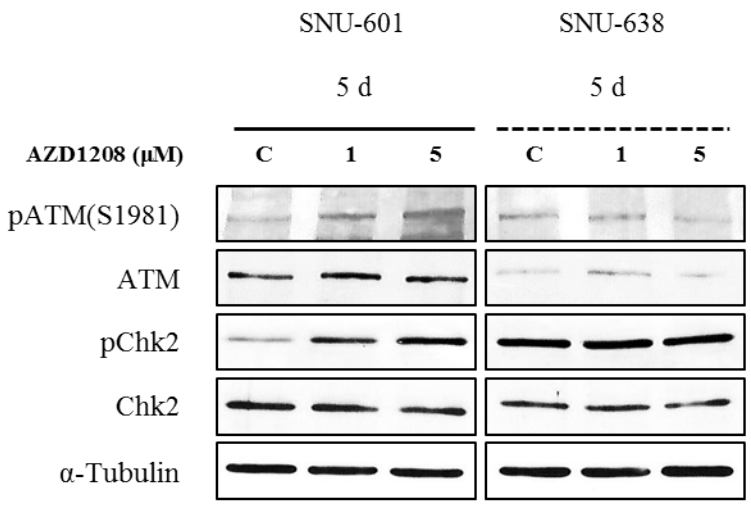



**Figure 7. DNA damage repair pathway is associated with resistance to AZD1208.** Cells were treated with DMSO and 1 or 5 μM AZD1208 for 120 h. Expression levels of ATM and Chk2 were measured by Western blot analysis.

## 6. Combined treatment with AZD1208 and an Akt inhibitor enhances antitumor effects and overcomes drug resistance in gastric cancer cells.

Overactivation of Akt signalling pathway has been detected in gastric cancer [26]. Pim can not only induce resistance to Akt inhibition, but Akt modulates DDR signaling through interaction with DNA damage sensors such as ATM, Ataxia Telangiectasia and Rad3-related protein (ATR), and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) [27]. Thus, we believed that co-administration of Akt and Pim inhibitors would exert more potent cytotoxic effects than treatment with each reagent alone. We therefore monitored the combined effects of Pim and Akt inhibition using a CFA. As expected, the percentage of growth inhibition for the gastric cancer cell lines observed with dual treatment was significantly lower than treatment with each reagent alone (Table. 3). In particular, the colony formation ability of AZD1208-resistant SNU-601 cells was dramatically reduced by the combined treatment compared to exposure to AZD1208 alone. These results suggested that dual inhibition of Pim and Akt synergistically blocked cell proliferation and could overcome resistance to AZD1208.

**Table 4. Combined effects of AZD1208 with an Akt inhibitor.**

| Cell line | Growth inhibitory rate (%, means±SD) | | |
|---|---|---|---|
| | AZD1208 (1μmol/L) | AZD5363 (500nmol/L) | AZD1208 + AZD5363 |
| SNU-216 | 16.2±11.1 | 15.3±4.9 | 47.2±4.7 |
| SNU-484 | 9.6±1.3 | 2±7.0 | 43.5±2.3 |
| SNU-601 | 21.1±5.8 | 23.1±1.2 | 65.1±8.8 |
| SNU-638 | 58.3±12.1 | 12.3±6.0 | 70.2±13.5 |
| SNU-668 | 29.8±5.6 | 12.6±8.8 | 25.9±14.9 |
| SNU-719 | 45.8±2.5 | 8.1±8.5 | 27.4±3.6 |
| AGS | 2.1±9.4 | 7.0±6.6 | 32.6±6.4 |
| MKN-45 | 22.9±8 | 1.6±6.5 | 24.3±11.2 |
| N87 | 18.3±10.2 | 13.8±5.3 | 59.2±11.4 |

The growth inhibitory activity of AZD5363 mono and dual inhibition with AZD1208 were measured with CFA. Cells were seeded and cultured with 500 nM of AZD5363 and 1 μM of AZD1208 every 3 d. The cells were cultured for 14 d until colonies formed and stained. $IC_{50}$ values were calculated and are represented in the table.

# DISCUSSION

Pim kinases are overexpressed in various types of tumors. Studies on the development of novel Pim kinase inhibitors have been published [15, 28]. In a previous investigation, it was reported that AZD1208 is a potent pan-Pim kinase inhibitor in cases of hematological cancer [29] but this effect had not yet been observed for gastric cancer. In the present study, we first assessed the effects of AZD1208 and underlying mechanisms of action in a gastric cancer cell line panel. AZD1208 could down-regulate the phosphorylation of Pim kinase substrates in a short period of time but did not induce apoptosis. Furthermore, AZD1208 induced cell death through autophagosome formation by LC3B in a long-term culture system. We also found that increased levels of DDR factors led to AZD1208 insensitivity. Moreover, we observed that a combination of AZD1208 with an Akt inhibitor had significant antitumor effects in the gastric cancer cell lines, suggesting that AZD1208 exerts therapeutic effect alone but also in combination with the Akt inhibitor.

Most studies of Pim inhibitors have shown that treatment with these reagents induces apoptosis through a reduction in Pim kinase substrates phosphorylation [14, 28, 30]. Another previous study also demonstrated

that AZD1208 triggers apoptosis in AML cells [29]. However, we did not observe apoptotic cell death although factors associated with cell survival, such as Bad and 4E-BP1, were down-regulated by Pim inhibition. On the other hand, it was reported that the Pim inhibitor SGI-1776 induces limited apoptosis and autophagy in cases of multiple myeloma although it was unclear how Pim inhibition caused autophagic cell death [12]. In our investigation, we determined not only that AZD1208 induced autophagic cell death but also the sensitivity of AZD1208 was associated with DDR capacity. Wang et al. noted that Pim-1 directly binds to and phosphorylates the CDK inhibitor p21 at $Thr^{145}$ [31]. It is well known that deregulated cell cycle progression causes replication stress and DNA damage [32]. Additionally, p53 induces autophagy in a damage-regulated autophagy modulator (DRAM)-dependent manner in the presence of DNA damage [33]. Based on the results of these studies, Pim inhibition by AZD1208 appears to disrupt progression of the cell cycle through the dysregulation of cell cycle components, resulting in increased levels of replication stress. Accumulation of DNA damage by Pim inhibition could also induce autophagy.

Activation of the ATM-Chk2 pathway was observed in SNU-601 cells following Pim inhibition. SNU-601 cells were found to have low levels of DRAM mRNA expression, which can affect the initiation of

autophagy (data not shown). Moreover, SNU-601 cells have PIK3CA mutation. These findings suggest that overactivated PI3K/Akt pathway induced DDR pathway and DNA damage induced by AZD1208 is proficiently repaired, resulting in resistance to AZD1208. Thus, we confirmed that autophagy induction could determine sensitivity to AZD1208 and alteration of PI3K pathway is associated with the resistance mechanism through DNA repair pathway.

Targeting the Akt signaling pathway has produced a limited antitumor effect due to drug resistance through a negative feedback loop and toxicity [21, 34]. Inhibition of Pim could overcome these problems and induce cell death to a greater extent. In our study, we observed that AZD1208 synergistically enhanced AZD5363 cytotoxicity through the growth inhibition in most gastric cancer cell lines. These results provide a rationale for administering a combination of Pim and Akt inhibitors to treat gastric cancer.

In summary, our report is the first to describe the effects of the Pim inhibitor AZD1208 on gastric cancer cells. Data from the study suggest that the Pim inhibitor can act as a sensitizer of an Akt inhibitor in gastric cancer cell lines. This could represent a potential strategy for treating gastric cancer. Furthermore, our findings provide a better understanding of the mechanism underlying the effects of Pim inhibition.

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# 국문초록

Pim kinase는 암세포의 증식과 생존 과정을 조절하는 serine/threonine kinase로서 위암을 포함한 다양한 인체 암종에서 Pim-1, Pim-2,Pim-3로 구성된 Pim 아형들의 발현이 높다고 보고되었으며 그 발현 분포가 암 종에 따라 다르다. AZD1208은 이러한 Pim kinase의 세가지 아형을 모두 억제할 수 있는 선택적인 억제제이다. 본 연구는 AZD1208에 의한 Pim kinase 활성 억제가 위암세포주의 세포 증식 억제에 미치는 영향과 기전을 알아보고자 하였다. AZD1208을 단독으로 처리하였을 경우, Pim kinase가 직접적으로 작용하는 기질들의 발현 양상이 감소하는 것을 관찰하였지만, 단기간으로 배양하였을 경우에는 암세포의 증식에 미치는 영향을 관찰할 수 없었다. 그러나, 장기간 배양하였을 경우, AZD1208에 민감한 일부 세포주 에서 세포 증식의 감소와 세포의 자가 소화작용이 관찰되었다. 또한, 세포의 자가소화작용의 표지자인 LC3B의 발현이 억제제의 처리 양에 비례하게 증가하는 것을 관찰 하였다. 이러한 현상을 통하여 AZD1208의 항암효과는 세포 자멸사가 아닌 자가소화작용을 통해 나타남을 확인하였

다. 뿐만 아니라, AZD1208에 저항성을 가진 세포 주에서 DNA 복구 기능에 중요한 역할을 하는 ATM과 Chk2 kinase의 발현이 활성화 되어있는 것을 통하여 DNA 복구 기능이 AZD1208의 항암 효과를 상쇄시키는 저항 기전이 될 수 있음을 제시하였다. 또한 Akt inhibitor와 함께 사용하였을 때 항암 효과가 증대됨을 확인하였다.

결론적으로, 본 연구에서는 위암 세포주에서 AZD1208이 자가소화작용을 활성화 시킴으로써 항암 효과를 보이는 것을 확인하고, Pim 억제제와 Akt 억제제를 병용 투여 하였을 때 더 효과 적인 항암효과가 있음을 제시하고자 하였다.

주요어: AZD1208, Pim kinase 억제제, 자가소화작용, 세포사, Akt, 위암

학번: 2012-23651