Effect of aurora kinase inhibitor binding mode on c-myc protein stability

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Finally I always gratefully thanks to my loving parents and brother who encouraged me, and my loving Bichon Frise Cco-cco who greeted me every day and night. Thanks a lot again, bless you with happiness.

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ABSTRACT

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c-Myc is a well-known transcription factor and known to be overexpressed in over 70% of cancers. So it is an important target oncogene, but it is considered as an undruggable target. Therefore various indirect regulating strategies of c-Myc have been studied. Among them, destabilizing the Aurora kinase A (AURKA)/c-Myc complex is considered as an important way to target c-Myc. As well as dual inhibition of c-Myc and AURKA has been shown to have more anti-tumor efficacy. In this study, I intend to conduct a study on how to regulate c-Myc through Aurora kinase inhibitors (AKIs), which is involved in c-Myc stability. Through the charge interaction study by using molecular docking, it was checked that the DFG (Asp-Phe-Gly) conformation of AKIs has correlated with c-Myc stability, by electrostatic attraction. It is finding of general correlation of AKIs DFG conformation and c-Myc downregulation , Type 1 (DFG-in) AKIs that bind to the active form of AURKA can degrade c-Myc, Type 2 (DFG-out) AKIs can't degrade c-Myc. This has been identified in several AML cells to check DFG-in AKIs degradation of c-Myc. Furthermore, by mechanistic study of c-Myc degradation induced by DFG-in AKIs, this effect showed due to UPS

degradation of c-Myc. And c-Myc induced by DFG-in AKIs showed lose of its function as transcription factor. Finally this study showed only DFG-in AKIs can degrade c-Myc in AML xenograft mouse model, daily orally treated mouse with DFG-in AKI had decreased c-Myc protein level.

Key words : c-myc, aurora kinase inhibitor, binding mode

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I. INTRODUCTION

c-Myc is one of the MYC family, the third most regulating factor in the genome[1]. It is a transcription factor, that is an elongated protein containing a basic helix-loop-helix (bHLH) and a leucine zipper (LZ)[2]. The LZ domain forms a heterodimer with MAX and activates the corresponding transcription target by binding to the E-box sequence[3].

In normal cells, c-Myc is well regulated as a transcription factor and regulates cell differentiation and proliferation through various mechanisms such as transcriptional amplification of target genes. However, in many tumor cells, c-Myc is overexpressed to plays a role in tumor formation, maintenance and differentiation[4]. Therefore, controlling and suppressing c-Myc is considered important in cancer therapy[5]. c-Myc determines over 70% of all kinds of cancer, and kill up to several hundred thousand cancer patients every year[6]. For example, many B-cell lymphomas and acute myeloid leukemia patients are depend on c-Myc for pathogenesis[7,8].

Unfortunately, c-Myc is considered an “undruggable target”[9]. Because as an transcription factor it is placed in the nucleus, many inhibitors mostly

inaccessible to target c-Myc. In addition c-Myc doesn't have a specific active sites to target due to its linear structure[10].

Recently, instead of directly inhibiting c-Myc, various strategies for indirect inhibition have been studied. Targeting c-Myc in transcription, mRNA translation, stability and Myc-MAX complex is researching. Among them, the most actively researched area is the strategy to destabilize the protein c-Myc, and many studies have been conducted to destabilize it through molecules such as ubiquitin-specific protease (USP) and Polo Like Kinase 1 (PLK1), especially Aurora kinase A (AURKA). As well as Some previous studies have demonstrated that AURKA overexpression were often accompained of c-Myc overexpression[11]. Therefore, dual-inhibition of c-Myc and AURKA would be an attractive therapeutic avenue for cancer[12].

AURKA is serine/threonine kinases that are crucial for a faithful transition through mitosis, and plays an important role in centrosome maturation, spindle assembly, meiotic maturation, and metaphase I spindle orientation[13]. AURKA shows oncogenic properties and in many cancers it has been found to be overexpressed such as breast, pancreatic, ovarian, gastric, colorectal and prostate cancers. Current studies showed that some Aurora kinase inhibitors (AKIs) has not only inhibit AURKA oncogenic functions, but also downregulates oncogenic c-Myc functions. It was reported that MLN8237 (Alisertib) inhibits cancer cell growth by proteasomal degradation of c-Myc complex with AURKA in NRAS-driven TP53-altered liver cancer cells[14]. In addition, there is a literature reporting that the treatment of Alisertib in thyroid cancer produces an antitumor effect due to the degradation of c-Myc[15]. As such, some AKIs are attracting attention due to their regulation of c-Myc, and their correlation with Asp-Phe-Gly (DFG) motif structures is being actively studied[16].

Before explaining DFG motif, kinases have N-terminal lobe and C-terminal lobe. Two lobes are connected by a flexible hinge region with ATP-binding site in the core of the protein[17]. The kinase activation loop which is crucial for kinase catalytic functions is located near hear, starting in DFG motif. Two major classes of conformation in kinase active sites are exist. When kinase is in active form, its activation loop opens by ASP of DFG motif interacting with an oxygen atom of the β phosphate of ATP. So it is often called as the open conformation kinase. But in inactive states of kinase, it doesn't need to activation loop activated to any catalytic activity[18].

In kinase inhibitor, it can be divided into two types of inhibitor according to the change in the motif of the kinase when it binds. Type 1 kinase inhibitors bind at the ATP site not allosteric pocket so it keeps the kinase in its active form. Type 2 kinase inhibitors contact both ATP site and adjacent allosteric site so it keeps the kinase in inactive form[19].

I thought that there would be a difference between AKIs known to be downregulate c-Myc and AKIs that were not, and this difference will be caused by the structure of the inhibitor described above. Therefore, I studied the correlation between the AKIs binding mode (DFG motif) and the stability of c-Myc.

First, the correlation between AKIs structure and the c-Myc stability based on the binding mode of the inhibitor was checked by molecular docking model. Then, by using a chemical biological approach, I tried to check whether it was applied to several cancer cells especially in Acute in acute myeloid leukemia cells. Furthermore, it was experimented that when DFG-in AKI was administered daily in a cancer xenograft mouse model, tumor western blot showed that c-Myc protein level was lower than vehicle mouse.

To summarize, the purpose of this study is to examine the effect of AKIs

binding mode on c-Myc protein stability by not only in the docking model, but also in several cancer cells and even in xenograft mouse model.

Through this study, the general correlation between conformation of AKIs and c-Myc stability was investigated. By my research, I wish this would be helpful to approaching new inhibitor to regulate c-Myc by AKIs.

II. MATERIALS AND METHODS

1. Cell culture

MOLM-14 and MV4-11 acute myeloid leukemia cells were cultured in RPMI (Welgene, gyeongsan, Korea, LM011-01) containing with 10% of FBS (Welgene, gyeongsan, Korea) and 1% antibiotics of penicillin and streptomycin. These cells were maintained in a humidified 5% Carbon Dioxide (CO_2) incubator at 37 ℃.

2. Cell proliferation assay (Cell titer Glo)

To obtain the GI50 value of the AKIs, cell viability was measured by using Cell titer glo (Promega, Madison, Wisconsin, USA, G7572) was used for MOLM-14. 5,000 cells/well were seeded and after 4 hours of stabilization of cells, inhibitors were treated. Inhibitors were treated for 10 points of 1:4 serial dilution (0–100 μmol/L). After 72 hours, each well's viable cells were normalized by well of 0.5% DMSO-treated wells (100%). Curve was fitted and GI50 value were calculated by Prism 7.0 software (GraphPad). This were performed twice, and three independent experiments were done.

3. Western Blot

To verify protein levels, every cells were pre-treated without or with indicated AKIs for indicated hours. Thereafter, cells were washed with 1X PBS Solution (Welgene, gyeongsan, Korea, LM011-02) and lysed with 1%

NP40 containing lysis buffer (50 mM Tris-HCl (pH 7.4), 1% NP40, 0.5 mM EDTA, 150 mM NaCl protease inhibitor cocktail tablet and phosphatase inhibitor tablet (Roche, #11–878-580–001 and #49-068-370-001, respectively). The protein concentration was measured via BCA assay (Intronbio, Seongnam, Korea, 21071), and western blot protein sample were made in same quantities. These sample were separated by 10% of SDS-PAGE, and then transfer on NC membrane (Amersham, Buckinghamshire, England 10600002). Immunoblotting analysis was done by using the following antibodies : c-Myc (D84C12) Rabbit mAb (Cell signaling Technology, Danvers, Massachusetts, USA, 5605), Phospho-c-Myc (Thr58) (E4Z2K) Rabbit mAb (Cell signaling Technology, Danvers, Massachusetts, USA, 46650), Ubiquitin (Cell signaling Technology, Danvers, Massachusetts, USA, 3936), Phospho-c-Myc (Ser62) (E1J4K) Rabbit mAb (Cell signaling Technology, Danvers, Massachusetts, 13748), Aurora A/AIK (1G4) Rabbit mAb (Cell signaling Technology, Danvers, Massachusetts, 4718), GAPDH (D16H11) XP® Rabbit mAb (Cell signaling Technology, Danvers, Massachusetts, USA, 5174), Phospho-Aurora A (Thr288) Monoclonal Antibody (Thermo Fisher scientific, Waltham, Massachusetts, USA, MA5-14904). All primary antibodies were used with diluted in TBS-T at 1:1000. Each primary antibody was incubated 3 hours for room temperature. Following the secondary antibody treated for 1 h at room temperature. Secondary antibodies were bought from genDEPOT (Houston, Texas, USA).

4. RT-PCR

Total RNAs was isolated from MOLM-14 by using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA) and 3 μg of total RNA was

prepared for qRT-PCR. RNA used to make cDNA by using M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA). The same concentration of cDNA was amplified by real-time quantitative PCR (qRT-PCR). qRT-PCR was performed by using the Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Following primer sequences in this study were used : for CAD (Homo sapiens carbamoyl-phosphate synthesize 2, aspartate transcarbamylase, and dihydroorotase , transcript variant 2, mRNA), 5-ACCACGACACCTGAAAGACC-3 (forward), 3-TACTGGTGGTGGAGGGTAGC-5 (reverse); for CDK4 (Homo sapiens cyclin dependent kinase 4 mRNA), 5-GAAACTCTGAAGCCGACCAG-3 (forward), 3-AGGCAGAGATTCACTTGTGT-5 (reverse); for ODC1 (Homo sapiens ornithine decarboxylase 1, mRNA), 5-CCCAGCGTTGGACAAATACT-3 (forward), 3-TCCATAGACGCCATCATTCA-5 (reverse); for CCND2 (Homo sapiens cyclin D2, mRNA), 5-TGGGGAAGTTGAAGTGGAAC-3 (forward), 3-ATCATCGACGGTGGGTACAT-5 (reverse); for SHMT2 (Homo sapiens serine hydroxymethyl transferase 2, mRNA), 5-CAGACTCAGACTGGGGAAGC-3 (forward), 3-GCAGAGGTGGTGGATGAAAT-5 (reverse).

5. Actinomycin D chase assay

To determine c-myc mRNA stability, 1 million MOLM-14 cells was seeded in 1 mL culture media and equilibrated in a humidified 5% Carbon Dioxide (CO_2) incubator at 37 ℃ for 4 hours. MOLM-14 were exposed to 1 μM of DMSO or AKIs for 15 minutes, followed by 5 μM actinomycin D (Sigma-Aldrich, Burlington, Massachusetts, USA, A1410) treatment. Then

MOLM-14 were harvested at every 20 minutes after actinomycin D addition. And RNA precipitation and qRT-PCR were performed as previously described.

6. Pulldown assay

For check the AURKA and c-Myc protein complex model, the MOLM-14 were pretreated with 5 mg/ml MG-132 (Calbiochem, Kenilworth, New Jersey, USA, 474790) for 4 hours with a 1 or 10 μM AKIs (ENMD-2076, CYC116, Alisertib, MK5108, MK8745) for 4 hours before lysis with cell lysis buffer. To see ubiquitination of c-Myc, the MOLM-14 were pretreated with 10 mg/ml MG-132, and following procedures are the same as before. Pulldowns performance were done with anti-c-Myc antibody (Cell signaling Technology, Danvers, Massachusetts, USA, #5605) and Protein G sepharose beads (Sigma-Aldrich, Burlington, Massachusetts, USA, P3296). Immunoblots (western blot) were performed as previously described.

7. Xenograft

MOLM-14 cells (2.5×10^6 cells/0.1 mL) were implanted into the right flank of 10-week-old female Balb/c nude mice (Orient Bio Inc.). When the tumor volume reached around 100 or 200 mm3, the tumor-bearing mice were separated into 2 cohorts (n = 2 per cohort). The vehicle substances are follows: 5% NMP, 15% solutol, 30% PEG400 and 50% 0.05M citric acid. Mice were orally gavaged once daily for Vehicle, ENMD-2076 for 7 or 9 days. The formula used to calculate tumor volumes is tumor volume = 1/2 (length × width × width). % of tumor growth inhibition (TGI) as calculated

by the following formula (1-{Tt/T0/ Ct/C0/1-{C0/Ct}}) X 100 , Tt = median tumor volume of treated at time t, T0 = median tumor volume of treated at time 0, Ct = median tumor volume of control at time t, C0 = median tumor volume of control at time 0.

8. Tumor western blot

1 ml of reagent was used for every 0.1 g tumor. Liver cells were homogenized at 30 Hz for 2 min using TissueLyser II (Qiagen, German). Using the lysate thus obtained, it was tested by the western blot method mentioned above. 1 ml of reagent was used for every 0.1 g tumor. Tumor were homogenized at 30 Hz for 2 min using TissueLyser II (Qiagen, German). Using the lysate thus obtained, it was tested by the western blot method mentioned above.

9. Fisher's exact test

To calculate the p-value, RStudio program were used. In MOLM-14 and MV4-11, when AKIs were treated for 1 μM and 4 hours, c-Myc protein level were obtained by quantified by GAPDH protein. AKIs that reduced c-Myc more than half compared with DMSO were judged to have degradation of c-Myc.

III. RESULTS

1. Calculation charge of AURKA's c-Myc binding pocket with molecular docking model

Before conducting a molecular biological experiment on the effect of AKIs binding mode on c-Myc protein stability, I tried to obtain the clue of the above experiment through molecular docking.

It is known that a specific amino acid has a specific charge, and some protein interaction structure are affected by charge[20]. This can be confirmed by molecular docking, and I tried to check the charge interaction when AURKA and c-Myc are interacting. Thankfully, Dr. Kim Namdoo performed molecular docking calculations (Figure 1).

Surprisingly, by docking structure of this two proteins (AURKA, c-Myc), inactive form (closed structure) of AURKA has positive charge at c-Myc binding portion. Before explaining why this is surprising, the life cycle of c-Myc in the cell, it undergoes phosphorylation before proteasomal degradation[21]. Since the phosphate group binds to c-Myc in this process, c-Myc immediately charge with anion before degradation. I got a huge hint from this. In Type 2 (DFG-out) AKIs which bind to the AURKAinactive form, AURKA has positive charge and willing to bind stronger with c-Myc which has an anionic[22]. This can explained with electrostatic attraction, the phenomenon where a negatively charged atom or molecule is attracted to a positively charged atom or molecule[23]. Finally Type 2 AKIs will give long life to c-Myc. In other words, in Type 1 (DFG-in) AKIs, that bind to the active form of AURKA, since there is no previously described phenomenon, it can be said that the binding force between two is weakened, resulting in lower c-Myc stability.

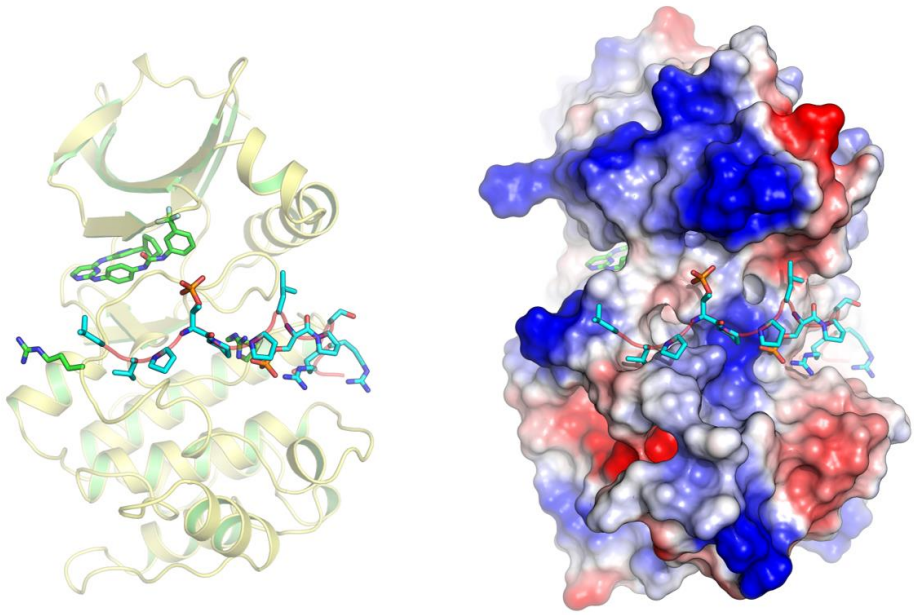


Figure 1. Predicted binding mode of inactive form of AURKA and c-Myc.

The negative charge is red part, and the positive charge is blue. Deeper color indicates a stronger trend.

2. Correlation of AURKA DFG conformation induced by AKIs and c-Myc degradation in AML (acute myeloid leukemia) cells

By docking study, I thought that general correlation of AKIs DFG conformation of c-Myc downregulation. I hypothesized that AKIs of DFG-in will able to degrade c-Myc in AML because active form of AURKA and c-Myc doesn't have charge interaction. But AKIs of DFG-out structure will have no c-Myc downregulation due to its electrostatic interactions. I tried check our hypothesis in acute myeloid leukemia (AML) cell line, which is known to increase mortality when c-Myc is overexpressed (Figure 2)[24]. To confirm this, I selected well studied 27 AKIs (Table 2)[25]. Also I choosed two AML cell, which are popularly being researched, MOLM-14 and MV4-11 (Figure 3 A,B). And finally find out general correlation of AKIs DFG conformation and c-Myc downregulation tendency.

Among 27 various AKIs, there are some compounds that do not conform to my general correlation, such as GSK-1070916. So I tried to checked whether this result is significant of not with the p-value. A p-value is a statistical measure used to test a hypothesis against the observed data, and p-values higher than 0.05 (> 0.05) are not statistically significant[26]. In this study, I used Fisher's exact test by Rstudio program[27]. Because Fisher's exact test are able to test the contingency table such as downregulation according to DFG conformation. By this test, protein western blot result and hypothesis has correlation with p-value of 0.0007 at MOLM-14, and 0.06 at MV4-11. So it was statistically confirmed that it had a significant correlation specially at MOLM-14 cells.

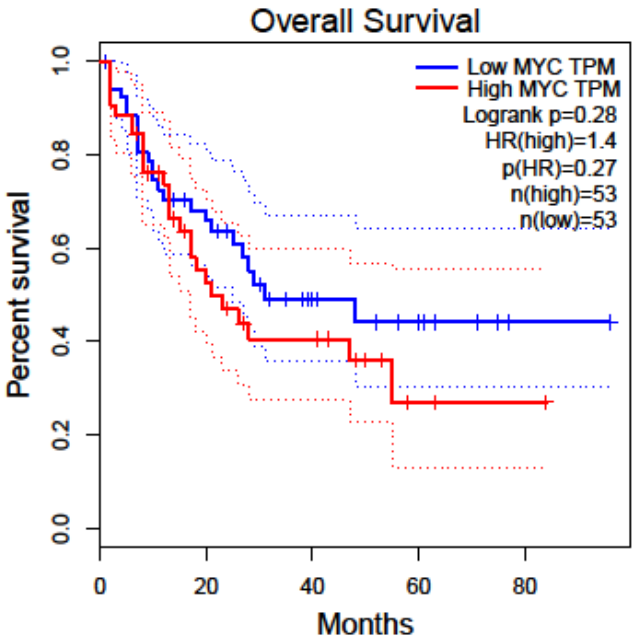


Figure 2. Overall survival of c-Myc in acute myeloid leukemia(AML) cancer patients.

This table is analyzed by GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses.

Table 1-1. The table shows selected data for the 16 DFG-in AKIs analyzed in this study.

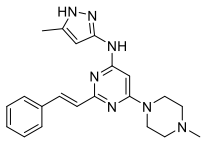
Aurora kinase intibitors	DFG-in/both/out by X-ray
ENMD-2076	in
SNS-314	in
Barasertib (AZD1152)	in
Staurosporine	in
CCT-129202	in
CCT-137690	in
CYC116	in
Hesperadin	in
JNJ-7706621	in
KW-2449	in
PF-03814735	in
Reversine	in
TAE-684 (NVP-TAE-684)	in
TAK-901	in
BI-847325	in
ZM-447439	in

Table 1-2. The table shows selected data for the 4 DFG-both and 7 DFG-out AKIs analyzed in this study.

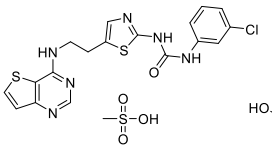
Aurora kinase intibitors	DFG-in/both/out by X-ray
AT-9283	both
Danusertib (PHA-739358)	both
Alisertib (MLN8237)	both
Tozasertib (VX-680)	both
PHA-680632	out
MLN8054	out
AMG900	out
GSK-1070916	out
LY3295668 (AK-01)	out
MK5108 (VX-689)	out
MK8745	out

Structural data of DFG conformation by x-ray is from the Protein Data Bank and references[28-33]. The table is broken into two sections corresponding to the three classes (DFG-in, both and out) of conformational described.

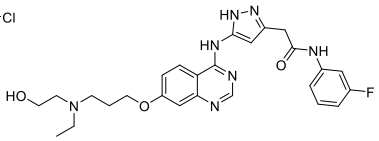
Table 2-1. The structure of selected 16 DFG-in AKIs.



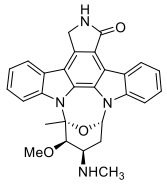
ENMD-2076



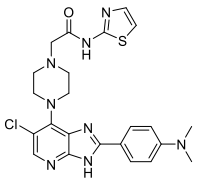
SNS-314



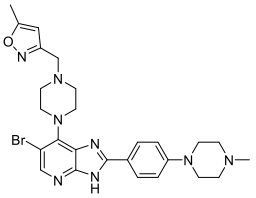
Barasertib (AZD1152)



Staruosporine



CCT-129202



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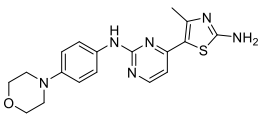
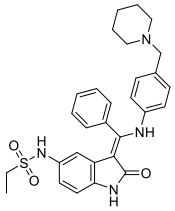
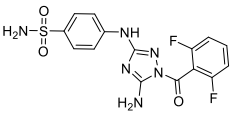


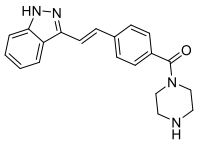
CYC116



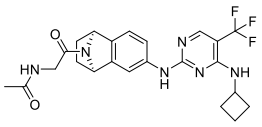
Hesperadin



JNJ-7706621



KW-2449



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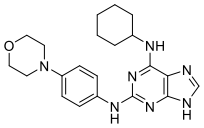
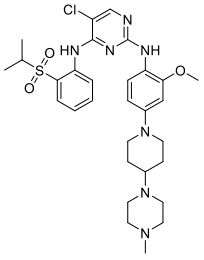
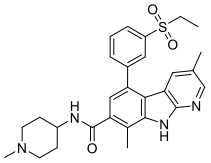


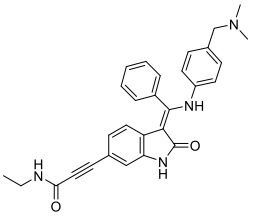
Reversine



TAE-684 (NVP-TAE-684)



TAK-901



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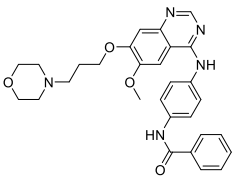
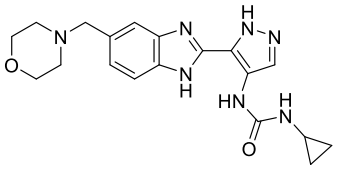
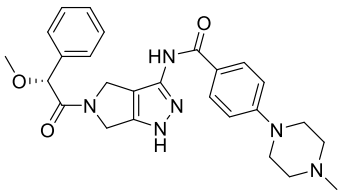


ZM-447439

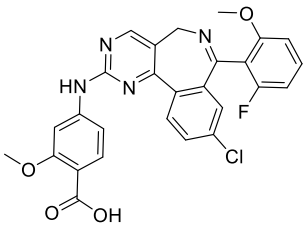
Table 2-2. The structure of selected 4 DFG-both AKIs.



AT-9283



Danusertib (PHA-739358)



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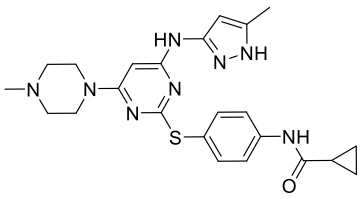
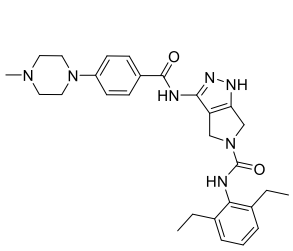


Tozasertib (VX-680)

Table 2-3. The structure of selected 7 DFG-out AKIs.



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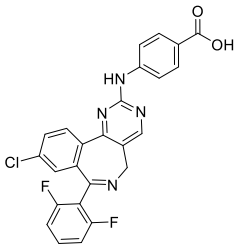

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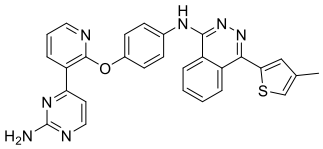

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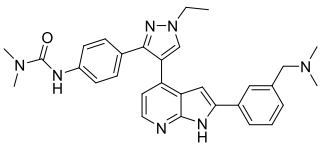

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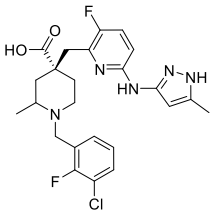

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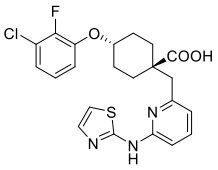

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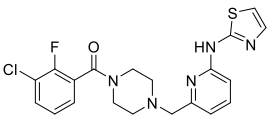

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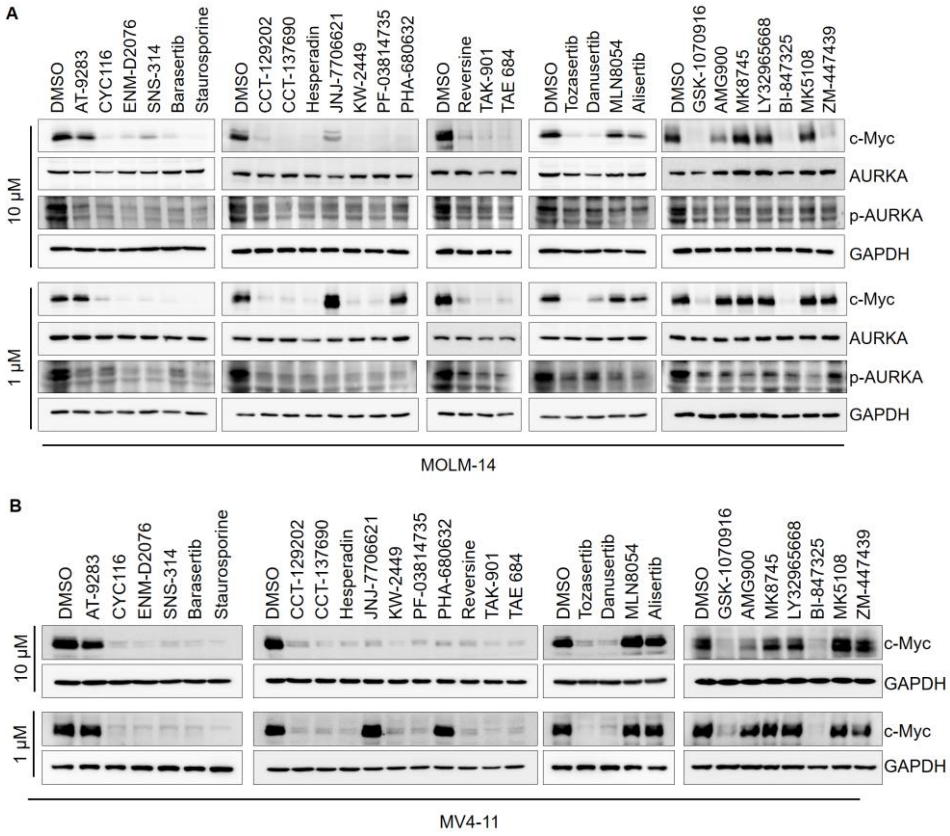


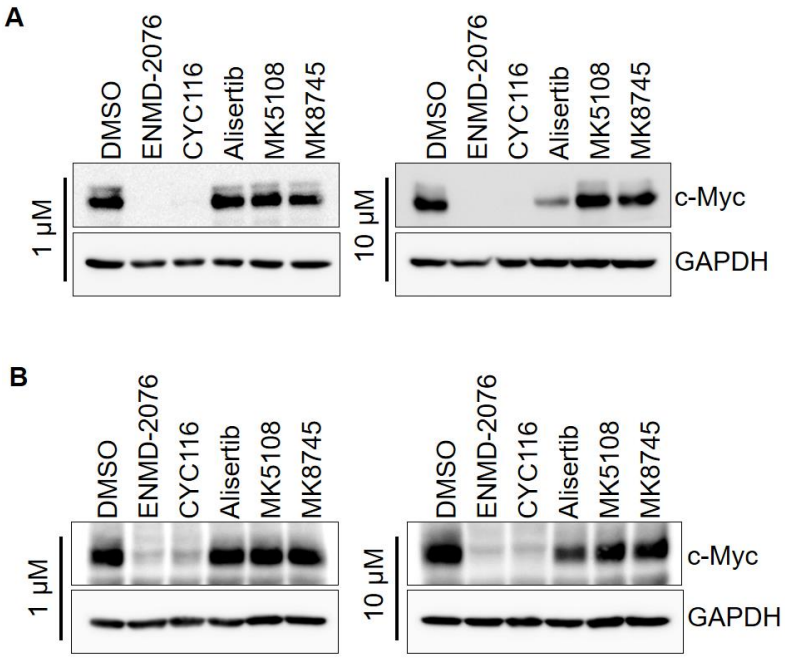
Figure 3. To find out general correlation of AKIs DFG conformation and c-Myc downregulation tendency, western blot of 27 selected AKIs was performed.

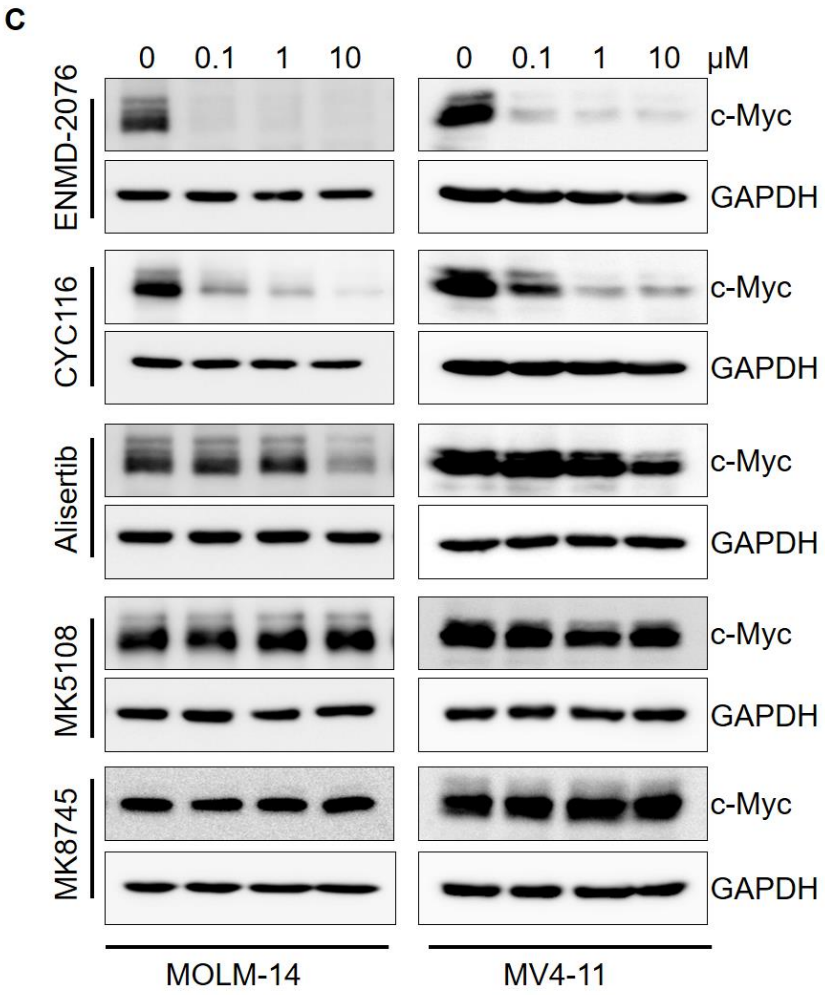
AKIs were treated at the indicated concentration for 4 hours (staurosporine were treated for 0.1 μM due to cell toxicity) in MOLM-14 (A) and MV4-11 (B). This were performed twice independent experiments.

Out of 27 AKIs, I wanted to selected representative AKIs for each DFG-in/both/out conformation based on the X-ray structure and fluorescence binding data by previous studies[28]. Finally I choosed ENMD-2076 and CYC116 for DFG-in AKIs, Alisertib for DFG-both AKIs and MK5108 and MK8745 for DFG-out AKIs.

To see correlation of DFG conformation of AKI and downregulation of c-Myc again in MOLM-14 (Figure 4 A) and MV4-11 (Figure 4 B), as I predicted ENMD-2076 and CYC116 which are DFG-in structure can degrade c-Myc but MK5108 and MK8745 which are DFG-out conformation can't degrade c-Myc.

And DFG-both conformation Alisertib can degrade c-Myc at higher concentration. In addition this downregulation of c-Myc by DFG-in AKIs showed for dependent on concentrations, but DFG-out AKIs showed no changes even longer times and higher concentration (Figure 4 C). Also unlike DFG-out AKIs, DFG-in and DFG-both AKIs showed a time dependence c-Myc downregulation for clearer (Figure 4 D).





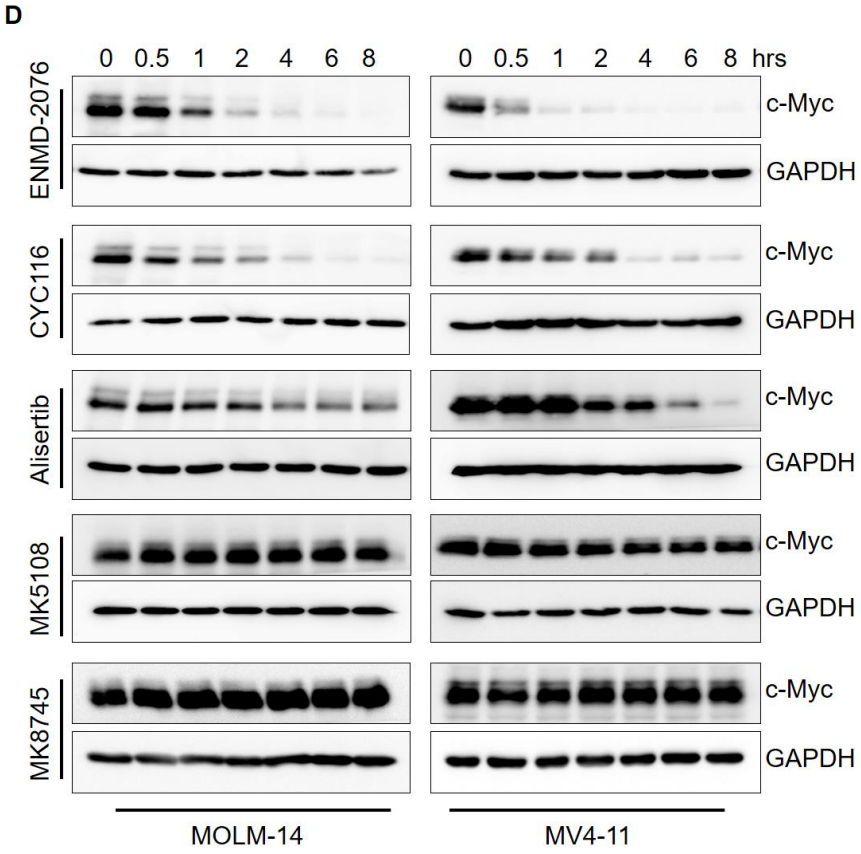


Figure 4. DFG conformation dependent c-Myc downregulation of representative AKIs of DFG-in, both, and out AKIs' at AML cells.

(A) MOLM-14 were treated at the indicated concentration for 4 hours. (B) MV4-11 were treated at the indicated concentration for 4 hours. (C) Each MOLM-14 and MV4-11 cells were treated for the increasing concentration for 4 hours. (D) Each MOLM-14 and MV4-11 cells were treated for the increasing hours for 10 μM, and for longest time, there was no cell cytotoxicity. This were performed twice independent experiments.

3. The effect of c-Myc by DFG-in AKIs is due to mechanism of ubiquitination proteomics system

I wondered if this phenomenon of c-Myc downregulation is due to the ubiquitination proteomics system. Because c-Myc is very unstable, so it was thought that this could occur at the mRNA level.

Through previous papers, c-Myc protein are known to be regulated by the proteasome, so I wanted to see mRNA level of c-Myc downregulation conditions[34]. I treated ENMD-2076, CYC116, Alisertib to MOLM-14 which showed downregulation of c-Myc, and c-myc mRNA half life were checked by Actinomycin D chase assay. After treatment with the transcriptional inhibitor actinomycin D, and analysis with quantitative real time-polymerase chain reaction (qRT-PCR), the half life of c-myc mRNA was obtained. As a result, the half life of DMSO treated MOLM-14 mRNA c-myc was about 36 minutes which is previously reported[35]. And AKIs treated MOLM-14 mRNA c-myc half life were showed a difference of less than 5% (Figure 5 A). Therefore, it was confirmed that c-Myc downregulation due to AKIs isn't effect of c-myc mRNA levels.

Next, I conducted three experiments to see this c-Myc downregulation is regulated by the proteasomal system. First, I treated MG-132, a proteasomal inhibitor, and I confirmed that c-Myc was rescued when MG-132 was treated with DFG-in AKI, in which c-Myc was degraded in the absence of MG-132 (Figure 5 B, C).

Second, I conducted a cycloheximide block experiment (Figure 5 D). The stability of endogenous c-Myc in DFG-in AKIs treated and untreated cells was checked at various time points after cycloheximide treatment. And as expected, the c-Myc half life was significantly reduced compared to

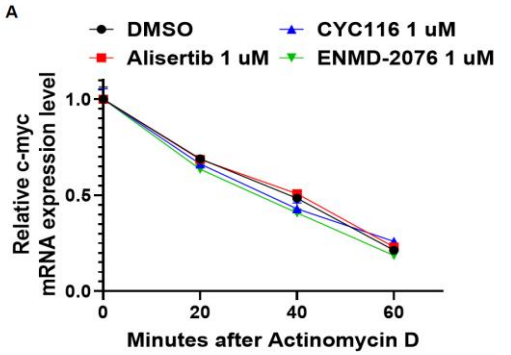
untreated cells.

Third, a sequential phosphorylation experiment of c-Myc degradation was conducted. Notably, c-Myc has an special life cycle. It is degraded through sequential phosphorylation steps[36]. First, Serine 62 (S62) is phosphorylated by DYRK2, and then Threonine 58 (T58) is phosphorylated by GSK3β. Then, S62 is dephosphorylated by PP2A and degraded by ubiquitination[37]. In the preceding literature, when Alisertib were treated in SF188 and GBM22 cells, at the total c-Myc degradation step, phosphorylation at S62 and T58 were confirmed. When total c-Myc is degraded, the phosphorylation of S62 was increased more than twice, and the phosphorylation of T58 was similar or slightly increased comparing with total c-Myc, confirming that these two phosphorylation forms were events that should precede c-Myc degradation[38]. In addition, when MYCi361 (small molecules that directly target c-Myc) were treated in PC3 cells, it was confirmed that T58 was increased more than 3 times compared to total c-Myc[39].

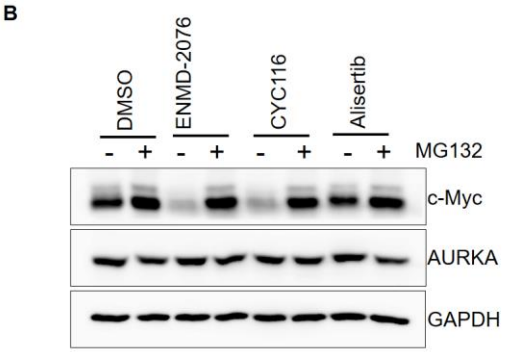
To checked these has correlation with our study, I treated ENMD-2076 and CYC116 and see the protein phosphorylation events preceding before c-Myc degradation (Figure 5 E). I obtained similar results to the previous study. Finally found that phosphorylation of c-Myc preceding before c-Myc degradation when DFG-in AKIs treated. As a result, I confirmed that c-Myc was degraded at the protein level when DFG-in AKI was treated.

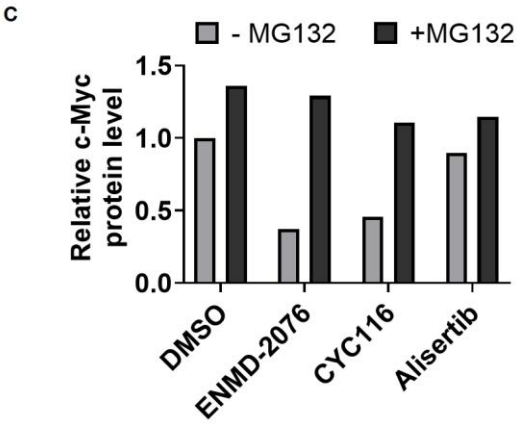
I thought that AURKA and c-Myc protein-protein complex contributed to the stabilization of c-Myc, and co-immunoprecipitation analysis was performed to confirm this. The result indicate that the AURKA/c-Myc complex was disrupted during DFG-in AKIs treated processing (Figure 5 F). This is supported by a previous study that when MYCN degraded AKI completely dissociates the MYCN-AURKA interactions[40].

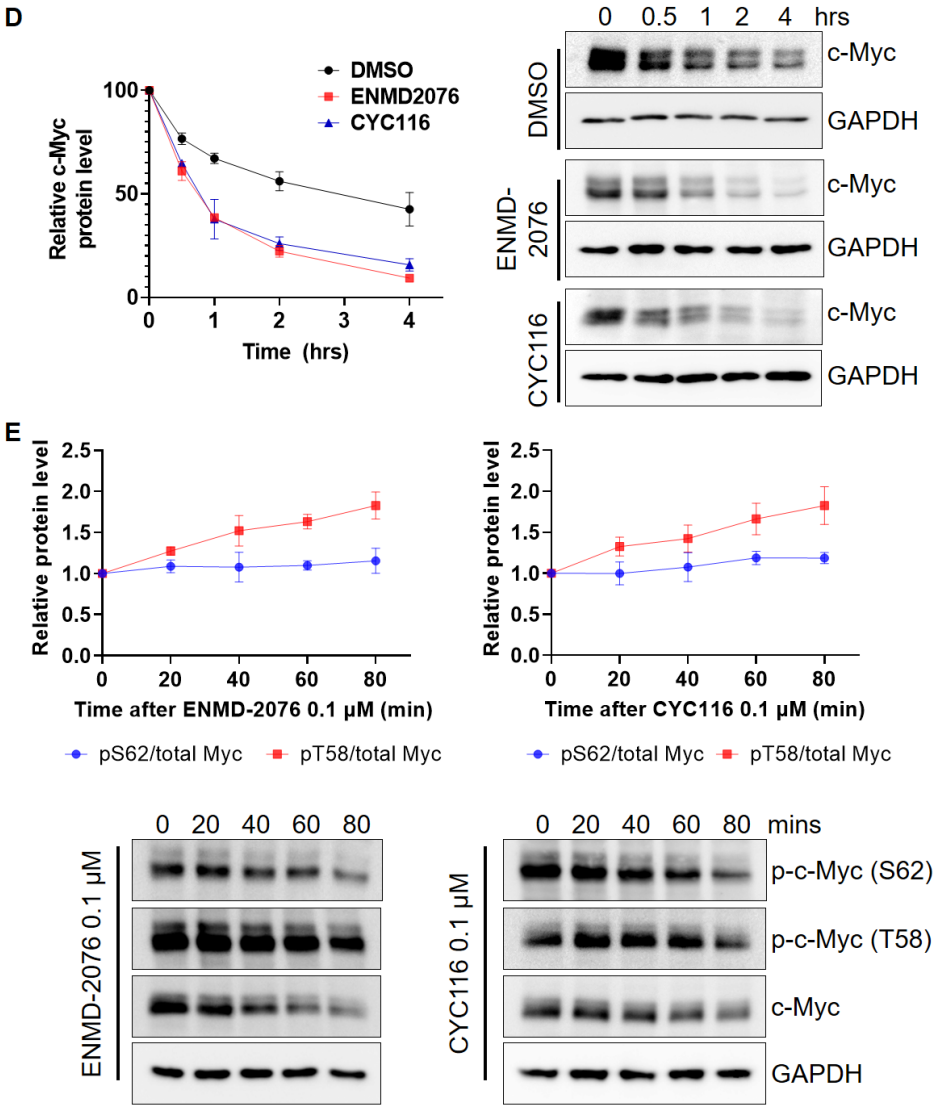
Based on these previous experimental results, I could infer that c-Myc degradation was promoted by increasing ubiquitination when DFG-in AKIs were treated. To check this AKI caused degradation of c-Myc is by promoting ubiquitination, immunoprecipitation was performed. MG-132 were pretreated to prevent c-Myc degradation, so ubiquitination of c-Myc rescue were seemed (Figure 5 G)[41]. At this condition, DFG-in and out AKIs were treated and then immunoprecipitate with c-Myc antibody and immunoblots with ubiquitin antibody. As a result, DFG-in AKIs treated sample were more ubiquitinated prepared with DFG-out AKIs treated sample (Figure 5 H). Through this experiment, it was found that degradation of c-MYC is due to promoting ubiquitination.



Intibitors	Linear slope	c-Myc half-life(min)	Ratio rel. control
DMSO	Y = -0.012X + 0.95	36.75	1
ENMD-2076	Y = -0.012X + 0.97	38.79	1.05
CYC116	Y = -0.012X + 0.96	37.88	1.03
Alisertib	Y = -0.013X + 0.96	36.16	0.98







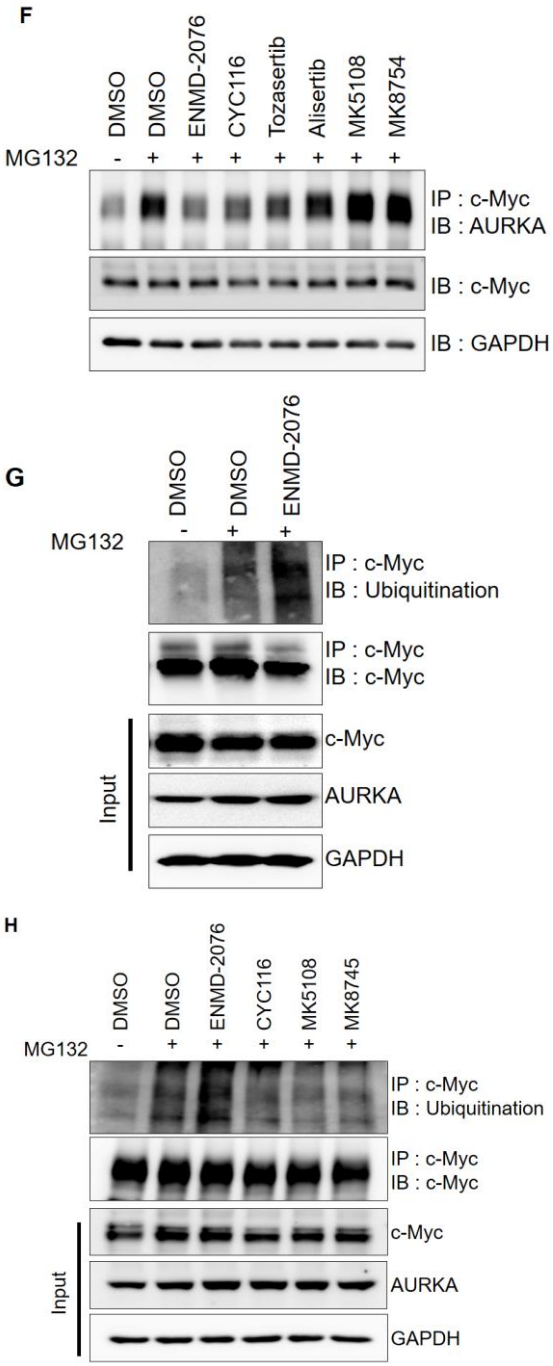


Figure 5. The effect of c-Myc by DFG-in AKIs is due to mechanism of ubiquitination proteomics system.

(A) To determinate c-myc half life, actinomycin D chase assay were experimented in MOLM-14. This were performed twice independent experiments. (B) With or without 5 μM of MG132 for 4 hours and treated with DMSO or 1 μM AKIs for 4 hours in MOLM-14. (C) The western blot band intensity of c-Myc in AKIs treated cells was normalized to that of GAPDH (housekeeping gene). (D) MOLM-14 were treated with 50 μg/ml CHX for 30 minutes, and AKIs were treated for indicated time, quantification of c-Myc levels are shown. This were performed three independent experiments. (E) Quantification of phosphorylated T58 and S62 c-Myc were normalized to that of total c-Myc in MOLM-14. This were performed three independent experiments. (F) Western blot of immunoprecipitation (IP) of c-Myc and total cell lysate (Input) in MOLM-14 cells treated for 4 hr with 5 μM MG-132 and for 4 hours with various 1 μM AKIs for 4 hours. (G,H) Western blot of immunoprecipitation (IP) of c-Myc ubiquitination and total cell lysate (Input) in MOLM-14 cells. MG-132 were treated for 10 μM for 4 hours and then AKIs were treated for 1 μM for 4 hours.

4. Mechanistic study for c-Myc degradation induced by DFG-in AKIs

As well known, c-Myc is regulated by various ubiquitin proteasome systems, and over a dozen of related ligases contribute to c-Myc ubiquitination and dedeubiquitination[39].

The known proteasomal system involved in ubiquitination consists of three enzymatic processes, which are composed of E1, E2, and E3. Among them, E3 ligase has the most diversity and is actively studied, and it is known that various E3 ligases such as FBXW7-α, SKP2, TRUSS, and PirH2 participate in the c-Myc ubiquitin proteasome system[42]. I wanted to find out whether the degradation of c-Myc was due to UPS when DFG-in AKIs were treated, and to confirm this degradation process mechanism by using E3 ligase and deubiquitinating enzymes (DUBs)[43].

First, it was attempted to examine SKP2, which is an important factor of c-Myc UPS. SKP2 is known to be an enzyme with unique properties that regulates c-Myc activation by binding to MB2 of c-Myc TAD and participating in degradation while also binding to HLH region[44]. When SKP2 is involved in c-Myc degradation, it acts as a component of Skp1-Cul1-F-box (SFC)-type E3 ubiquitin ligase, and bind to the F-box domain of Skp2 and establish association with SKP1.

The stability of the c-Myc protein was confirmed by treatment with blocking SZL-P1-41 (known as compound 25), and it was confirmed that the SKP2-SKP1 interaction was inhibited and the mechanism of c-Myc degradation was blocked (Figure 6 A). I was able to confirm that c-Myc protein was rescued when DFG-in AKIs was treated after disrupting SKP2-SKP1 interaction (Figure 6 B). It was confirmed that the c-Myc degradation by AKIs was caused by the UPS decomposition.

The ubiquitination of c-Myc can be removed by deubiquitination, and the

stability of c-Myc is controlled by several DUBs. A representative of them is USP28, which is a molecule that counteracts with FBXW7-α and contributes to the stabilization of Myc. It is well known that treatment with AZ1, known as an inhibitor of USP28, lowers the protein stability of c-Myc[45]. I confirmed that the protein stability of c-Myc was lowered when AZ1 was treated (Figure 6 C). After treatment of AZ1, I checked changes of c-Myc with DFG-in/out AKIs. With DFG-in AKIs, which had degradation, with pretreatment with AZ1, they had much more degradation of c-Myc (Figure 6 D). And with DFG-out AKIs, which had no degradation of c-Myc, with pretreatment with AZ1, they had increased degradation of c-Myc (Figure 6 E). From these results, it can be confirmed that the degradation of c-Myc by AKIs is through UPS again.

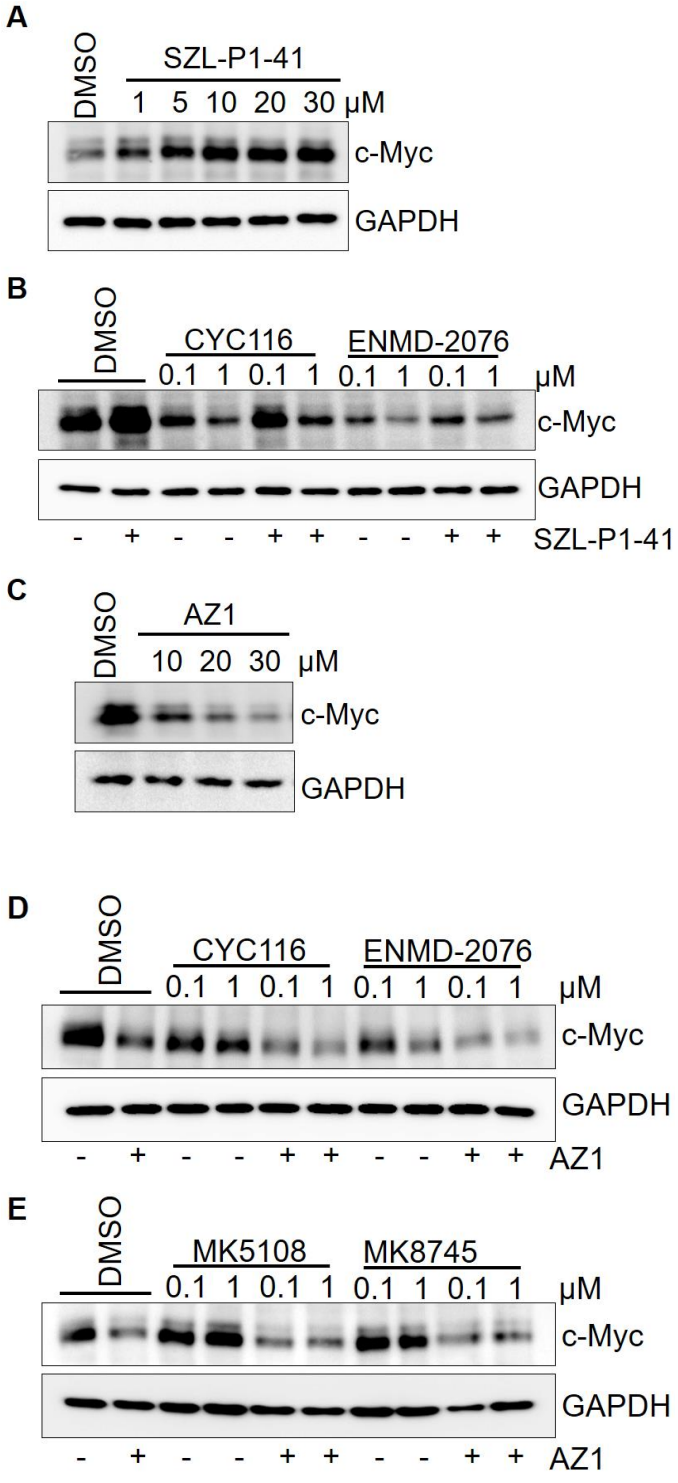


Figure 6. Mechanistic study for c-Myc degradation induced by DFG-in AKIs.

(A) MOLM-14 were treated with SZL-P1-41 for indicated concentration for 24 hours. (B) 5 μM SZL-P1-41 were pretreated for 24 hours in MOLM-14, and then DFG-in AKIs were treated for 2 hours for increasing concentrations. (C) MOLM-14 were treated with AZL for indicated concentration for 4 hours. (D) 30 μM AZ1 were pretreated for 3 hours in MOLM-14, and then DFG-in AKIs were treated for 1 hours for increasing concentrations. (E) Pretreated condition is same with (D), and then DFG-out AKIs were treated for 1 hours for increasing concentrations.

5. Mechanistic study of c-Myc degradation effects on its function

As mentioned before, c-Myc is a super transcription factor and regulates the transcription of various genes[46]. Representatively, among the target genes of c-Myc, the most well-accepted ones that are well known as important factors in metabolism are ornithine decarboxylase (ODC), serine hydroxymethyl transferase 2 (SHMT2) and carbamoyl phosphate synthase aspartate transcarbamylase dihydroorotase (CAD)[47-49]. I checked the mRNA expression levels of ornithine decarboxylase (ODC), serine hydroxymethyl transferase 2 (SHMT2) and carbamoyl phosphate synthase aspartate transcarbamylase dihydroorotase (CAD) under conditions of c-Myc degradation by treatment with DFG-in AKIs. Experimental results showed that the mRNA expression level of ornithine decarboxylase (ODC) (Figure 7 A), serine hydroxymethyl transferase 2 (SHMT2) (Figure 7 B) and carbamoyl phosphate synthase aspartate transcarbamylase dihydroorotase (CAD) (Figure 7 C) was significantly reduced compared to DMSO under the condition that c-Myc was degraded by treatment with DFG-in AKIs. But DFG-out AKIs that could not degrade c-Myc did not.

In addition to metabolism-related genes, among the gene related to the cell cycle, there are cyclin dependent kinase 4 (CDK4) and cyclin D2 (CCND2), which are known as target genes of c-Myc[50,51]. As a result of confirming the mRNA level, we were able to obtain similar results to those related to metabolism (Figure 7 D, E). Therefore, it was confirmed in several genes that the expression level of the target genes was lowered.

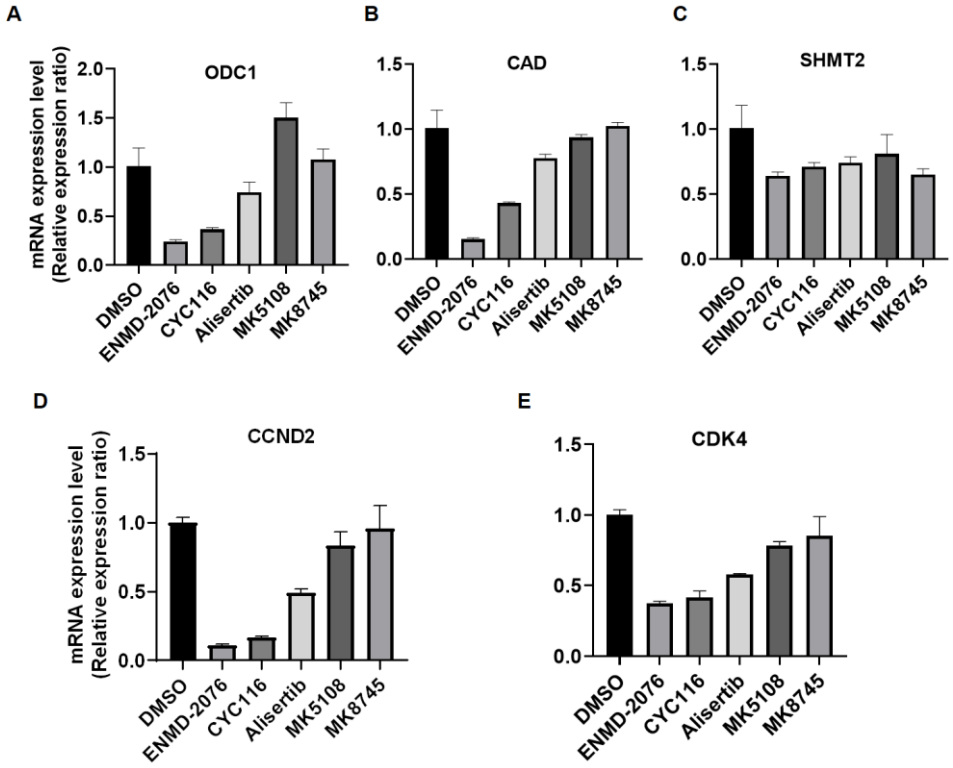


Figure 7. Mechanistic study of c-Myc degradation effects on its function by qRT-PCR.

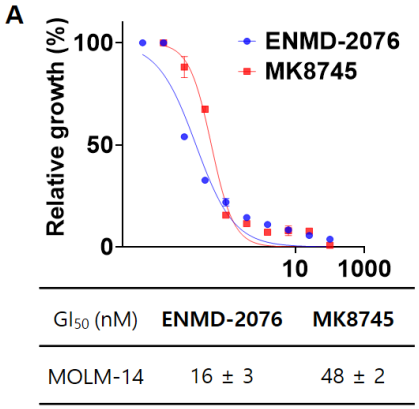
To establishment of MOLM-14 cell c-Myc target gene expression by qRT-PCR analysis. For (A) ornithine decarboxylase (ODC), (B) serine hydroxymethyl transferase 2 (SHMT2), (C) carbamoyl phosphate synthase aspartate transcarbamylase dihydroorotase (CAD), (D) cyclin D2 (CCND2) and (E) cyclin dependent kinase 4 (CDK4). This were performed twice independent experiments.

6. In vivo efficacy of AKIs in MOLM-14 xenograft model

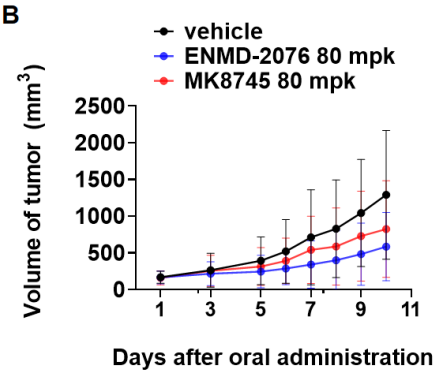
So far, I have tested this general correlation of AKIs DFG conformation and c-Myc degradation in molecular docking and cancer cells. After then, I tried MOLM-14 xenograft to see if this applies to the actual mouse model.

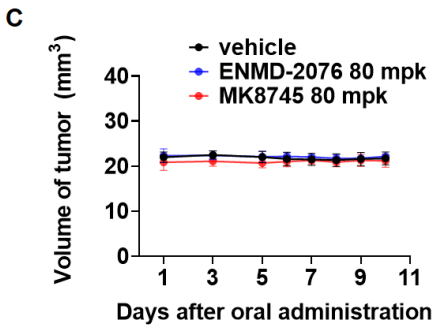
GI50 values and references were referred to determine the dose at which the TGI was sufficiently reduced[52]. GI_{50} values in MOLM-14 are 0.016 μM for ENMD and 0.048 μM for MK8745 (Figure 8 A). The balb/c nude mice bearing MOLM-14 xenograft tumor were grouped in 3 cohorts (vehicle, ENMD-2076 80 mpk, MK8745 80 mpk) randomly and each group are orally administrated daily. Tumor growth of MOLM-14 xenograft mouse, ENMD-2076 treatment group are remarkably delayed and MK8745 treatment groups are gradually delayed compared to the vehicle treatment group (Figure 8 B). Orally gavage was performed every day until the average tumor size of the vehicle reached 1500 mm3, and it was administered for a total of 10 days. There was no toxicity of AKI as there was no weight loss (Figure 8 C). While the average tumor weight of vehicle treatment group is about 1.3 g, the average tumor weight of ENMD-2076 was 0.3 g and 1.1 g for MK8745 treatment group (Figure 8 D).

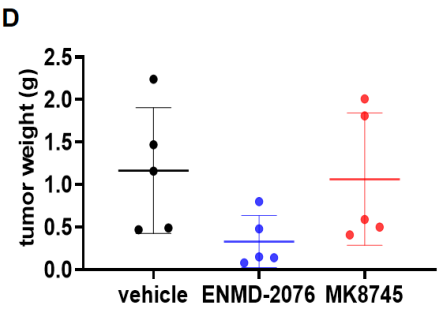
Through the above results, it was checked that AKIs were effective in mice administered with the two compounds. Then I tried to see the c-Myc protein level in tumor tissue. Like in cancer cell line, tumor western blot showed general correlation of AKIs DFG conformation and c-Myc degradation (Figure 8 E). Only in DFG-in AKI ENMD-2076 treated group decreased c-Myc protein with significant p-value of one-way ANOVA (Figure 8 F).

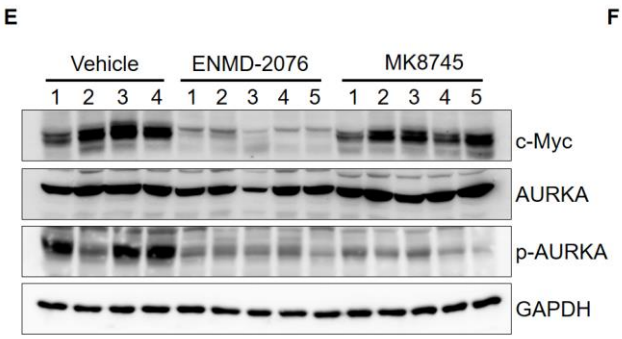


GI_{50} (nM)	ENMD-2076	MK8745
MOLM-14	16 ± 3	48 ± 2









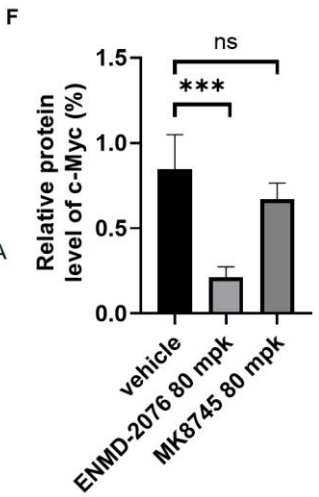


Figure 8. In vivo efficacy of AKIs in MOLM-14 xenograft model.

(A) Anti-proliferation activity of ENMD-2076 and MK8745 on MOLM-14. Cell viability of MOLM-14 after 72 hours exposure with two AKIs were determined with Cell Titer Glo experiments. This experiments are triplicate with independently. (B) to (E) Immunodeficient mice were Subcutaneous administrated with 5 x 106 MOLM-14 cells. (B) ENMD-2076, MK8745 (80 mpk ; mg per kg) were orally administered every day. After 10 days of oral gavage, two AKIs treatment delayed tumor growth. (C) Body weight was monitored every days during the experiments. There were no body weight loss. (D) Tumor weight of mouse were measured after sacrifice. (E) Western blot were experimented to check the protein level of tumor tissue. (F) Relative protein levels of c-Myc of MOLM-14 xenograft mouse tumor. Data are expressed with 0.0001 of P-value.

IV. DISCUSSION

Many studies of c-Myc in cell cycle, protein biogenesis, cell adhesion, metabolism, signal transduction, transcription, and translation. Among them, many are struggling to solve oncogenic c-Myc due to its role of cancer amplification, but there is no inhibitor yet. Because as a transcription factor, c-Myc doesn't have a specific active site for small molecules. This makes difficult to functionally inhibit c-Myc activities. Second, c-Myc is mostly located in the nucleus, so targeting nuclear c-Myc is technically impractical. To overcome these obstacles, strategies to indirectly regulate c-Myc oncogenic functions have been investigated. There is some promising approach, controlling c-Myc stability by Aurora kinase.

The Aurora kinases are key regulators of mitosis. Recently, some research groups showed that AKI Alisertib can act as c-Myc downregulator. Also others showed its derivative MLN8054 disrupt the Myc-AURKA complex, resulting c-Myc degradation and tumor regression. These data suggest that some structures of AKIs may be potential regulator for the treatment of c-Myc in cancer. By earlier research, this study could start with wondering if structural similarity may have correlation with c-Myc regulation.

At first, using molecular docking study of how AURKA and c-Myc structures fit together, inactive form of AURKA had positive charge at c-Myc binding portion. Before degradation step, c-Myc is phosphorylated by an anionic phosphate group. So Type2 AKIs can make AURKA and c-Myc protein complex more tightly, but Type1 AKIs couldn't.

Next, using 27 AKIs western blot to see this works in AML cell lines, which is known to increase mortality when c-Myc is overexpressed. Data show general correlation of AKIs DFG conformation of c-Myc

downregulation. In addition in ENMD-2076, CYC116, Alisertib, their downregulation was increased as time and concentration increased.

By earlier studies, c-Myc stability is known to tightly realated by the ubiquitin-proteasome system. And this research showed c-Myc degradation by DFG-in AKIs is caused by not mRNA level but protein levels. By blocking proteasomal inhibition system with MG132, c-Myc rescue were checked when treated with DFG-in AKIs. Moreover, before c-Myc degradation, pS62 c-Myc form was increase. And this were due to AURKA and c-Myc protein-protein complex disrupted by DFG-in AKIs.

Furthermore, mechanistic research of process and action of c-Myc degradation were done. By blocking SKP2, one of E3 ligase of c-Myc degradation UPS, with SZL-P1-41, c-Myc were rescued. Also, by blocking USP28, one of DUP of c-Myc degradation UPS, with AZ1, c-Myc was more degradable. When c-Myc was degraded by DFG-in AKIs, c-Myc function as a transcription factor was also losted when target gene expression level were checked by qRT-PCR.

This effect were shown in MOLM-14 xenograft mouse model. By orally administrated daily for 10 days, only DFG-in AKI ENMD-2076 degrade c-Myc, not in DFG-out AKI MK8745.

There are several additional points to consider of this thesis.

First, this thesis is only a limited number of cancer cell lines. Currently, several cell lines have been tried, but only MOLM-14 and MV4-11 hold this thesis, and MOLM-14 is well-known for highly dependent on MYC for leukemic maintenance[53]. Yet, there would be better to check anti-cancer effect by c-Myc degrade in c-Myc dependency cancer cell line. HL-60 is well-known for c-Myc dependent AML cell, but this didn’t showed effect of AKIs binding mode on c-Myc protein stability[54]. Due to this limitation of cell line, later it would be nice to find a cell line that works in other than

AML, and learn the genomic characteristics of cell lines that work with this thesis.

Second, among the 27 AKIs used in the experiment, there are some inhibitors that does not match with this thesis. There are a total of three compounds to mention. Two inhibitors, JNJ-7706621 and PHA-680632, showed degradation depending on the treated concentration and hours. But GSK-1070916 which has a structure of DFG-out, this AKI has downregulation of c-Myc not depends on concentration and hours. It was difficult to determine the exact progress of GSK-1070916 downregulation of c-Myc. It should be good to check the c-Myc binding site of GSK-1070916 combined AURKA has a positive charge through molecular docking. Also additional mechanism research should be studied by molecular biological experiments. Above all, kinase inhibitors do not have a complete DFG-in or out structure. Therefore, it would be study to indicate how strongly AKIs have DFG-in or out structure.

Despite these exception some AKIs, by contingency table (count matrix) Fisher's exact test I got p-value of less than 0.05. So this thesis reliably reports of general correlation of AKIs DFG conformation and c-Myc downregulation.

Lastly, a group recently reported research results about AKI that can degrade c-Myc and n-Myc[33]. They developed a novel AKI through structure-based drug design (SBDD). Their rational was that AURKA's DFG-out scaffold are highly potent. So their rational design were based on making DFG-out AKIs, and they reported this final AKI can degrade c-Myc and n-Myc simultaneously. However, they checked this effect in SCLC (NCI-H82, NCI-H446, NCI-H211, NCI-H524, NCI-H526, NCI-H146, NCI-H841, and NCI-H209) and neuroblastoma (SK-N-BE) cells, not in

AML cells. So direct comparison is difficult.

Nevertheless, this study showed effect of AKIs binding mode on c-Myc protein stability. In summary, this research found general correlation of AKIs DFG conformation and c-Myc downregulation. Type 1 (DFG-in) AKIs that bind to the active form of AURKA can degrade c-Myc, Type 2 (DFG-out) AKIs that bind to the inactive form of AURKA can't degrade c-Myc due to electrostatic attraction between positive charge AURKA's c-Myc binding pocket and negative charge of phosphorylated c-Myc. This were checked in molecular docking, cancer cell line and even in vivo experiments. So it can be helpful when designing AKIs to treat patients with both AURKA and c-Myc overexpression AML patients.

V. CONCLUSION

1. By calculation charge of AURKA with molecular docking, found general correlation of AKIs DFG conformation and c-Myc downregulation.
2. Type2 (DFG-out) AKIs can stabilize c-Myc due to electrostatic attraction but Type 1 (DFG-in) AKIs can't resulting lower c-Myc stability.
3. The effect of c-Myc degradation by DFG-in AKIs is due to mechanism of ubiquitination proteomics system. This were confirmed by following experimental conclusions. When DFG-in AKIs were treated, there is no change in c-myc mRNA half life, c-Myc is rescued from blocking proteasomal degradation pathway, c-Myc half life is reduced, the pT58 c-Myc increased before protein level degradation, and finally when immunoprecipitation with AURKA, c-Myc complex is reduced.
4. Degradation c-Myc by DFG-in AKIs is due to UPS. When block E3 ligase, c-Myc didn't degraded under conditions in which it was degraded. Similarly, when block DUP, c-Myc degraded under conditions in which it wasn't degraded.
5. Degradation of c-Myc caused lack of c-Myc function. By checking target gene expression level by qRT-PCR, we performed 5 target gene ODC, SHMT2, CAD, CCND2, CDK4. By treatment of DFG-in AKIs, c-Myc was degrade and finally these target gene expression levels were also lowed.
6. In vivo efficacy test of AKIs in MOLM-14 xenograft model demonstrated that DFG-in AKIs can degrade c-Myc.

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ABSTRACT(IN KOREAN)

Aurora 키나아제 저해제의 결합 모드와 c-Myc 단백질 안정도의 상관성 연구

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c-Myc는 잘 알려진 전사인자로 암의 50% 이상에서 과발현되는 것으로 알려져 있다. 따라서 c-Myc은 항암을 위해서 중요한 표적유전자 및 표적단백질이고, 특히 c-Myc의 과발현으로 인해서 여러 암환자들은 생존율이 감소하다고 잘 알려져 있기 때문에, 중요한 표적으로 알려저 있다. 그러나 c-Myc을 타겟하는 것은 여러 이유 때문에, 직접 타겟이 불가능한 표적으로 간주되고 있다. 따라서 c-Myc을 간접적으로 조절할 수 있는 여러 연구가 진행되고 있으며, 그 중 오로라 카이네이즈와 c-Myc의 단백질 복합체를 불안정하게 하며 최종적으로 c-Myc을 표적으로 하여 저해/분해 시키는 전략이 활발히 연구 되어지고 있다. 본 연구에서는 c-Myc의 안정성에 관여하는 오로라 카이네이즈 저해제들을 통해 c-Myc을 조절하는 방법에 대한 연구를 수행하고자 한다. 오로라 카이네이즈 에이와 c-Myc의 결합 도킹 모델을 통해 전하 상호작용을

확인하였고, 이를 통해 오로라 카이네이즈 에이의 DFG (아스파테이트-페닐알라닌-글라이신) 형태가 c-Myc의 조절과 상관관계가 있음을 확인하였다. Type 2의 오로라 카이네이즈 저해제들을 DFG-out (불활성 상태, 닫힘) 구조의 오로라 카이네이즈 에이에 결합하게 되는데, 이때 오로라 카이네이즈 에이의 결합부위는 양전하를 띄고, 분해직전 c-Myc은 음전하를 띄게 되므로 정전기적 인력을 통해 이 복합체는 안정화가 됨을 확인하였다. 반면에 Type 1 오로라 카이네이즈 저해제들을 위의 현상이 없으므로 c-Myc이 불안정해져 최종적으로 분해가 된다. 도킹 모델뿐 아니라 분자생물학적 실험기법을 통해 이것이 다양한 세포내에서 적용이 됨을 됨을 확인하였다. c-Myc이 분해되는 과정들이 UPS를 통한 단백질 분해이며, c-Myc이 분해될 때 전사인자로서의 기능이 상실함을 여러 기전연구를 통해 확인하였다. 또한 MOLM-14 세포주를 이식한 마우스 모델에서, DFG-in AKI를 투여한 실험군에서 c-Myc이 감소됨을 확인하였다. 본 연구를 통해 DFG-in 구조의 오로라 카이네이즈 저해제는 오로라 카이네이즈의 catalytic 기능을 저해할 뿐만 아니라, c-Myc을 동시에 저해 (분해) 할 수 있다는 일반적 상관성을 규명하였다.

핵심되는 말 : c-myc, 오로라 카이네이즈 저해제, 결합모드