약학석사학위논문

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2016년 8월

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김 지 현

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이 논문을 약학석사학위논문으로 제출함

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약학과 의약생명과학전공
김지현

김지현의 석사학위논문을 인준함
2016 년 6월

위 원 장 (인)
부 위 원 장 (인)
위 원 (인)

ABSTRACT

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PD0332991, also known as Palbociclib, is a specific inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, which got accelerated approval by Food and Drug Administration in 2015 for estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer patients. Unlike pan CDK inhibitors, PD0332991 is less toxic due to its specificity to CDK4/6, and doubled progression-free survival in metastatic breast cancer when combined with letrozole. However, PD0332991 treatment unexpectedly stabilized Cyclin D3 and CDK4/6 complexes and emergence of CDK4/6 mutation is highly expected based on the experiences of other kinase inhibitors.

In the previous study, we proved that methionyl-tRNA synthetase (MRS) controls the stability of CDK4 by directly interacting with CDK4. MRS is an essential enzyme, which links methionine (Met) to $tRNA^{Met}$ doing a critical role in global translational regulation, but meanwhile, it facilitates cell proliferation via stabilizing CDK4, especially in $p16^{INK4a}$-negative cancer. Inhibition of MRS by small interfering RNA or Met analogue treatments reduced CDK4 level resulting in cell cycle arrest at G0/G1.

Based on this, I screened about 200 compounds to identify specific MRS-CDK4 interaction inhibitors, which can control CDK4 with different mode of action from that of PD0332991. I investigated the effects of the compounds on the cell proliferation, MRS activity and CDK4 level and finally found BC-MCI-CG-88 compound. BC-MCI-CG-88 directly hindered the interaction between MRS and CDK4 in immunoprecipitation assay and induced cell cycle arrest at G0/G1 suppressing cell proliferation. Moreover, it did not inhibit the catalytic activity of MRS implying that it would deliver its cytotoxic effects to only $p16^{INK4a}$-negative cancer, but not to normal cells or tissues. This study is meaningful in that it suggests a novel possible therapeutics to target CDK4 via inhibiting the interaction between MRS and CDK4, which can be applicable to $p16^{INK4a}$-negative cancer and hopefully, to PD0332991-resistant $p16^{INK4a}$-negative cancer.

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keywords : MRS (Methionyl-tRNA synthetase), CDK4 (cyclin-dependent kinase 4), $p16^{INK4a}$ (cyclin-dependent kinase inhibitor 2A), methionine analogue, cell-cycle inhibition

Student number : 2014-25280

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Ⅰ. INTRODUCTION

Cyclin-dependent kinase 4 (CDK4) is a cell cycle regulator responsible for G1-S transition, activating cell cycle signaling by forming a complex with cyclin D. CDK4-cyclin D complex releases E2F via phosphorylating Retinoblastoma (Rb), and free E2F increases the transcription of cell cycle-related proteins which are required for the S-G2 transition, accelerating the cell proliferation [1]. Under normal conditions, there are endogenous CDKs inhibitors such as INK4 and KIP/CIP-types and they control CDKs timely and accurately to maintain a balanced cell cycle [2-4].

Among the CDKs inhibitors, $p16^{INK4a}$ is a specific CDK4 inhibitor, and plays a crucial role in cell-cycle regulation by inhibiting CDK4 and attenuates cell cycle progression from G1 to S phase. In various cancers, $p16^{INK4a}$ is commonly mutated, deleted or inactivated by hyper-methylation on the promoter region [5]. When $p16^{INK4a}$ is mutated or deleted, spontaneous tumorigenesis goes faster and it shows poor prognosis in most cases compared to $p16^{INK4a}$-positive cancers [6-9].

Recently, attentions have been paid to CDKs-targeted therapies due to the FDA approval for Palbociclib [10]. Palbociclib, also known as PD0332991, is one of the CDK4/6 inhibitors. Unlike other pan CDK inhibitors, targeting CDK4/6 is less cytotoxic without affecting gene transcription [11, 12] and more potent in that CDK4 or CDK6 can replace the function of CDK2 [13-15]. Actually, PD0332991 treatment with letrozole enhanced progression-free survival of metastatic breast cancer patients in PALOMA-1 trial [16] and other clinical trials are in progress to extend its applications to other types of cancer [17, 18]. On the other hand, there is some skepticism towards the efficacy and potency of PD0332991. While PD0332991 significantly slowed the progression of advanced cancer, it did not show statistically significant results in the overall survival rates of patients [19, 20]. In addition, there was a report that

PD0332991 treatment paradoxically increased the stability of cyclin D3-CDK4/6 complex [21]. Moreover, PD0332991 is a kinase inhibitor, therefore resistance occurrence based on the acquired mutations on CDK4/6 by treatment of PD0332991 is expected as observed in other kinase inhibitors [22, 23]. Considering that CDK4 is a validated target, which is critical for oncogene-dependent transformation and mammary tumorigenesis [3, 24-27], it is valuable to try other strategies to find novel anti-CDK4/6 therapeutics, which works differently with PD0332991.

Aminoacyl-tRNA synthetases (ARSs) are enzymes, which provide charged tRNAs for translation by attaching specific amino acids to their cognate tRNAs. Besides their role in translation, ARSs are involved in diverse signal pathways which are critical for various diseases including cancer [28]. Methionyl-tRNA synthetase (MRS) is one of ARSs that loads methionine to $tRNA^{met}$. In the previous study, we found that MRS stabilizes CDK4 by binding to CDK4 when $p16^{INK4a}$ is inactivated. Knockdown of MRS by small interfering RNA (siRNA) decreased the level of CDK4 resulting in cell cycle arrest at G0/G1 [29]. We also identified a methionine analogue, Fmoc-Sec(Mob)-OH (FSMO), which hinders the interaction between MRS and CDK4 at relative low concentrations and affects the catalytic activity of MRS at relative high concentrations. When cells were treated with low concentrations of FSMO, CDK4 and MRS were dissociated each other and the CDK4-stabilizing effect of MRS was diminished resulting in cell-cycle arrest at G0/G1. FSMO was effective only in the $p16^{INK4a}$-negative cancer cells but not in normal cells and $p16^{INK4a}$-positive cancer cells. It is worth noting that FSMO showed the possibility of developing MRS-CDK4 interaction inhibitors, which do not affect the catalytic activity of MRS.

The purpose of this study is to investigate specific MRS-CDK4 interaction inhibitors, which would be effective and suitable for treating $p16^{INK4a}$-negative cancer patients. Based on the several screening procedures, I found BC-MCI-CG-88, which

showed selective cytotoxicity to $p16^{INK4a}$-negative H460 cancer cell line compared to WI-26 normal cell line. It revealed better cytotoxic effect on H460 cancer cell than PD0332991 in the same concentration. Also, unlike FSMO, BC-MCI-CG-88 had no effect on the canonical function of MRS, which is crucial for normal cell survival. In this paper, I present the novel compound, BC-MCI-CG-88, which can control the stability of CDK4 via dissociating CDK4 from MRS. It has the potential to be applicable to p16 ^{INK4a}-negative cancers with less cytotoxicity in normal cells and tissues.

Ⅱ. MATERIALS AND METHODS

1. Materials

The primary antibodies against MRS (Abcam, mouse, ab50793), CDK2 (Cell Signaling, rabbit, 78B2), CDK4 (c-22: Santa Cruz Biotechnology, rabbit, sc-260), CDK7 (Cell Signaling, mouse, #2916), Cyclin D (Millipore, rabbit, 04-221), β-actin (Sigma Aldrich, mouse, A1978), Myc (Santa Cruz Biotechnology, mouse, sc-40), HA-probe (F-7: Santa Cruz Biotechnology, sc-7392), Flag (Sigma Aldrich, F3165-1MG), Strep-HRP (IBA, 2-1509-001), and p16 (Santa Cruz, rabbit, sc-468) were used in this study. The anti-mouse antibody (Thermo Fisher Scientific, Host Goat-Anti, Antigen Mouse IgG [H+L], Cat.31430), and the anti-rabbit antibody (Thermo Fisher Scientific, Host Goat-Anti, Antigen Rabbit IgG [H+L], Cat.31460) conjugated with HRP were used as secondary antibody. Purified human $p16^{INK4a}$ protein (SRP3134) was purchased from Sigma Aldrich.

2. Cell culture

WI-26 and HeLa were purchased from either the American Type Culture Collection or the Korean Cell Line Bank, and maintained using DMEM (HyClone) supplemented with heat-inactivated 10% defined fetal bovine serum (FBS) and 50 μg/ml penicillin/streptomycin. HCC1937, MDA-MB-436, MDA-MB-231, MDA-MB-468, A549, H460, HCT116, and SK-BR-3 were purchased from the Korean Cell Line Bank and cultured using RPMI (HyClone) supplemented with heat-inactivated 10% defined FBS and 50 μg/ml penicillin/streptomycin. All cells were cultured in 5% CO_2 at 37℃.

3. Western blot

All cells were lysed by lysis buffer (50 mM Tris-HCl of pH 7.5, 0.5 % Triton X-100, 5 mM EDTA, 10 % glycerol, 150 mM NaCl, and protease inhibitor) for 30 minutes at 4℃. Then, the lysates were collected in 1.5 mL EP tube and centrifuged at 13,200 rpm for 15 minutes at 4℃. The supernatant proteins were quantified by Bradford assay (BioRad, Cat. 500-0006). Final samples were made by adding 5X Laemmli sample buffer and lysis buffer. Samples were boiled for 7 minutes to denature the proteins before gel running. After that, samples were loaded on SDS page gels and separated by electrophoresis. Proteins at the gel were transferred to polyvinylidene fluoride (PVDF) membranes purchased from Millipore (IPVH 00010) for 15 minutes, 25 V, 13 mA per gel. Each membrane was incubated with 5 % skim milk for 1 hour to block non-specific protein bindings. After removing the blocking solution, primary antibodies were added to the membrane during overnight at 4℃. Each membrane was washed with 0.5 % Tris-Buffered Saline-Tween (TBS-T) buffer for 5 minutes. Washing was repeated 3 times. After this process, secondary antibodies were added for 1 hour, and washed equally as it was done with the previous step. ECL solution (Santa Cruz or EZ-Western Lumi) was applied depend on each target proteins.

4. DNA transfection

X-tremeGENE™ HP DNA Transfection Reagent (Roche Life Science) was used to transfect plasmids. Preparation was performed as the manufacturer's instruction. After diluting plasmid DNA and reagent with 1:3 ratio at serum free DMEM media, pipetting the mixture was done to make the homozeneous mixture. After incubating for 15 minutes, the mixture was added to cells with fresh media and the cells were incubated in the incubator for 4 hours. After that, the media was changed to the RPMI complete media. Cells were harvested after 24 hours.

5. Immunoprecipitation (IP)

Cells were treated with BC-MIC-CG-88 or dimethyl sulfoxide (DMSO), and the interaction between MRS and CDK4 was investigated via IP. Cells were lysed with IP buffer (50 mM Tris-HCl of pH 7.5, 250 mM NaCl, 0.5 % NP40, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4 and protease inhibitor) for 30 minutes. Then, the cells were scrapped to collect lysate and centrifuged for 13200 rpm, 15 minutes to obtain supernatant. Protein extracts were incubated with the antib-CDK4 for 2 hours at room temperature, and then further incubated with the addition of protein agarose beads for 2 hours. After incubation, the beads were washed 3 minutes for 3 times with the IP buffer, mixed with SDS sample buffer and boiled to elute the proteins. Samples were separated by SDS-PAGE gel to detect each protein. .

6. Purification of yeast MRS protein

MBP (Myelin basic protein)-tagged yeast MRS plasmid, pMalC2-MBP-MRS, was kindly provided from Dr. Hubert D. Becker (University of Strasbourg, France). *Escherichia coli* (*E. coli*) strain harboring pMalC2-MBP-MRS was cultured at 37℃ and MBP-MRS was expressed by induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 25℃ overnight. Cells were disrupted by sonication in the reaction buffer containing 250 mM Tris HCl (pH 8.0), 100 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 5% glycerol, 10% β-mercaptoethoanol and protease inhibitors. The cell lysate was spun down for 30 min at 17,000 rpm and the supernatant was incubated with amylose resin (Clontech). The proteins bound to the resin were eluted with modified sonication buffer, with 300 mM NaCl and 0.36% maltose without Tween 20. The fractions with high purity were used for the aminoacylation reaction.

7. *In vitro* aminoacylation assay

All aminoacylation assays were performed at 37℃ with the reaction buffer containing 50 mM HEPES (pH 7.4), 20 mM potassium acetate, 10 mM magnesium acetate, 4 mM ATP, 2 mg/ml yeast tRNA, 25 μCi [35S] Met (PerkinElmer, 1000 Ci/mmol) and 10 μg of purified yeast MRS. Chemicals were treated at 100 μM concentration for 3 minutes. Aminoacylation reactions were quenched on the 3 MM filter paper, pre-wetted with the 5% trichloroacetic acid (TCA) solution. After washing with the 5% TCA solution and drying the paper, radioactivity was detected with the liquid scintillation counter (PerkinElmer).

8. Bromodeoxyuridine (BrdU) incorporation

H460 cells were seeded in 96-well plates at a density of 3000 cells per well. Next day, cells were treated with chemicals at 10 μM for 6 hours or DMSO as a control in 5% serum RPMI media. After 72 hour-incubation, 100 μl of media with 5% serum supplemented with BrdU was added and incubated for 2 hours. Cells were fixed and denatured with the solution in the BrdU cell proliferation assay kit (Cell Signaling) for 30 minutes at room temperature. Then, the solution was removed and prepared detection antibody solution was added and incubated for 1 hours at room temperature. The plates were washed three times with washing buffer and reacted with 100 μl/well of Horse Reddish Peroxidase (HRP)-conjugated secondary antibody solution and incubated for 30 minutes at room temperature. Then, the plates were washed 3 times with the washing buffer and 100 μl of tetramethylbenzidine (TMB) substrate was added and incubated for about 30 seconds at room temperature. The amount of BrdU incorporated into the DNA, was detected by measuring the absorbance at 450nm by ELISA reader.

9. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

H460 and WI-26 cells were seeded in 96-well plate at the density of 3000 cells per well. After 24 hours of incubation, cells in each well were treated with chemicals for 72 hours with 5% serum media. Cells were incubated with 20 ul of MTT solution (Sigma Aldrich, M2181, 5 mg/ml) for 2 hours. After removal of the media, 100 ul of DMSO was added to each well to dissolve MTT-formazan crystals. The absorbance was measured at 570nm wavelength.

10. Cell viability analysis using IncuCyte.

H460 cells were plated in a 96-well plate at a density of 2000 cells per well. After 24 hours of incubation, media were changed with 5% serum RPMI. Cells were treated with DSMO, 10 μM BC-MCI-CG-88 or 10 μM PD0332991 for 72 hours. The IncuCyte Kinetic Live Cell Imaging System (Essen BioScience) was used to monitor the cell growth rate.

11. Fluorescence-activated cell sorting (FACS)

After the treatment of DMSO or BC-MCI-CG-88 for 6 hours, H460 cells were harvested by trypsinization. Then the cells were washed twice, with the cold PBS, and fixed with 70% ethanol for 2 hours at 4℃. After the fixation, the cells were washed twice with the cold PBS and incubated with 500 μl propiodium iodide (PI, Sigma Aldrich) staining solution (100 μg/ml RNase A and 50 μg/ml PI in 1X PBS) for 30 minutes at 37℃ in the dark condition. The samples were then analyzed by flow cytometry (BD Biosciences). The percentages of cells in the G0/G1, S, and G2/M phases were analyzed using the Cell Quest acquisition software (BD Biosciences).

Ⅲ. RESULTS

1. FSMO attenuates cancer cell proliferation via blocking MRS-mediated CDK4 stabilization in $p16^{INK4a}$-negative cancer

In the previous study, we proved the importance of MRS for the stabilization of CDK4. When MRS was knocked-down by si-MRS in $p16^{INK4a}$-negative cancer, CDK4 got unstable and cell-cycle progression was halted. The same result was observed when cells were starved with Met or treated with Met analogue. Therefore, I used FSMO (Fmoc-Sec(Mob)-OH) (Figure 1A), identified as a Met analogue in our previous study, to confirm whether it can block the interaction between MRS and CDK4. FSMO treatment decreased the association between MRS and CDK4 in a dose-dependent manner (Figure 1B). FSMO treatment also reduced cell proliferation based on the BrdU incorporation assay (Figure 1C), suggesting that FSMO affected cell proliferation via inhibiting MRS-CDK4 interaction. Since MRS-mediated CDK4 regulation was clear in $p16^{INK4a}$-negative condition, we knocked down the level of $p16^{INK4a}$ in WI-26 cells, which are $p16^{INK4a}$-positive normal cells. As expected, FSMO clearly reduced CDK4 level without affecting CDK2, only when the level of $p16^{INK4a}$ was reduced by si-p16 transfection (Figure ID). The effect of FSMO and its $p16^{INK4a}$ dependency was also investigated in other cell lines (Figure 1E-F), showing that FSMO specifically inhibited the interaction between MRS and CDK4 in the cancer cells whose $p16^{INK4a}$ was inactivated.

2. Screening strategy to identify novel MRS and CDK4 interaction inhibitors

A total of 213 compounds were provided by Crystal Genomics, laboratory of

Professor Jeewoo Lee (Seoul National University) and laboratory of professor Gyoonhee Han (Yonsei University). These compounds are consisted of derivatives of FSMO, Met analogues, and possible MRS catalytic inhibitors and the latter was designed *in silico* based on non-mammalian MRS structures. Since these chemicals are not random library and designed specifically to target MRS, I decided to perform a phenotype-based assay as the primary screening (Figure 2A). The purpose of primary screening was to sort out the chemicals that would be cytotoxic to $p16^{INK4a}$–negative cancer cells compare to normal cells. I performed MTT assay as a primary screening using H460 and WI-26 cells and selected 8 chemicals. For the secondary screening, I carried out immunoblotting to investigate the effect of selected compounds on CDK4 level. I also confirmed the effect of cell proliferation based on BrdU incorporation assay (Figure 2A). Because BrdU can be incorporated into the newly replicated DNA of proliferating cells during S phase, decreased signal would be an indication of halted CDK4 signaling. Dose dependent effect of cell proliferation was also investigated.

To further validate the specific mechanism of compound selected by secondary screening, immunoblotting and *in vitro* MRS enzyme assay were performed. Based on the results, I chose BC-MIC-CG-88, which significantly decreased the CDK4 level without affecting MRS activity. The effect of BC-MCI-CG-88 on cell-cycle and cell growth were confirmed via FACs assay and IncuCyte. BC-MCI-CG-88 was used to confirm $p16^{INK4a}$ dependency on $p16^{INK4a}$-negative and positive cancer cells (Figure 2A).

3. Selection of compounds specifically inhibiting the proliferation of $p16^{INK4a}$-negative cancer

In order to conduct MTT assay as primary screening, H460, $p16^{INK4a}$–negative lung cancer cell line and WI-26, a $p16^{INK4a}$–positive normal lung cell line were chosen to investigate the efficacy and the toxicity of chemicals. DMSO was used as a negative

control and PD0332991 was used as a positive control. Cell viability was analyzed using MTT assay, treating 10 μM of chemicals for 72 hours. The selection criterion of compounds was choosing ones which showed below 60% of H460 proliferation to select more potent compounds than FSMO which revealed 67% of H460 proliferation at 50 μM. In addition, I chose compounds, which showed over 80% of WI-26 proliferation in that PD0332991 just allowed 65% of WI-26 proliferation when 10 μM of PD0332991 was treated (Figure 2B). Based on these criteria, compounds which are more cytotoxic to cancer cells than FSMO, and safer to normal cells than PD0332991 could be selected. As a result, I finally selected 8 chemicals based on MTT assay. Among the compounds, 3 were FSMO derivatives and 5 were *in silico* designed MRS interactors (Figure 2C). Their effects on the proliferation of H460 and WI-26 cells were compared showing that BC-MCI-CG-88 was the most toxic to H460 without any significant cytotoxic effect on WI-26 (Figure 2D).

4. BC-MCI-CG-88 was the most potent compound which reduced cell proliferation by destabilizing CDK4

To sort out the chemicals whose cytotoxicity to H460 is dependent to MRS-mediated CDK4 regulation, the effects of each 8 compounds on the level of CDK4 and on cell proliferation were investigated. Among 8 chemicals, BC-MCI-CG-79 and BC-MCI-CG 88 showed the most dramatic effect on CDK4 and Cyclin D levels (Figure 3A). Since it is known that Cyclin D is vulnerable to degradation without CDK4, it is not surprising that Cyclin D level was affected by these compounds [30]. Also, cell proliferation based on BrdU incorporation assay was affected by BC-MCI-CG-88 treatment (Figure 3B). Next, I investigated the dose dependent effect of these compounds on the H460 and WI-26 cells proliferation. The IC_{50} values of these compounds were calculated based on MTT assay (Figure 3C). These compounds were

selectively toxic to H460 cells as shown in the primary screening (Figure 1C-1D), and as expected, all the 8 compounds showed relatively low IC_{50} values in H460 cells (Figure 3C). Among them, BC-MCI-CG-88 was the most effective compound which inhibited 50% of H460 cells at the concentration around 3.99 μM (Figure 3D), and moreover, it is more effective in killing H460 than PD0332991.

5. BC-MCI-CG-88 interferes MRS-CDK4 interaction without disturbing MRS catalytic activity

BC-MCI-CG-88 (Figure 4A) was structurally independent to that of FSMO and I further investigated the mode of action of BC-MIC-CG-88 to see whether its cytotoxic effect on H460 would be derived from MRS inhibition or not. I confirmed again the effect of BC-MIC-CG-88 on H460 cells via live cell growth monitoring by IncuCyte analysis (Figure 4B). BC-MCI-CG-88, compare to PDO332991, effectively inhibited the real time cell growth. Next, the effect of BC-MCI-DG-88 on the MRS and CDK4 interaction was examined via immunoprecipitation (IP). H460 cells were transfected with Flag-tagged CDK4 and Strep-tagged MRS, and were treated with BC-MCI-CG-88. Cells were also treated with MG132 to prevent the proteasome-dependent degradation of CDK4. Flag-CDK4 was immunoprecipitated using anti-Flag antibody, and the dose-dependent effect of BC-MIC-CG-88 on MRS and CDK4 association was monitored. As the concentration of BC-MCI-CG-88 was increased, MRS-CDK4 binding was increasingly disrupted (Figure 4C).

Since BC-MCI-CG-88 was proved as the MRS and CDK4 interaction inhibitor, its effect on cell cycle arrest was also checked. CDK4 is important for G1-S cell cycle transition [1], therefore it was expected that BC-MCI-CG-88 would arrest cell cycle at G1. As shown in the figure, BC-MCI-CG-88 increased the portion of G1 phase cells showing cell cycle arrest at G1 (Figure 4D).

As mentioned previously, one of the demerits of FSMO is that it inhibits MRS catalytic activity although the required concentration is relatively high. Modulation of MRS catalytic activity may cause some toxic effect on normal cells. To see whether BC-MCI-CG-88 also has the ability to affect MRS activity, I did the *in vitro* enzyme assay using purified yeast MRS protein and found that BC-MCI-CG-88 did not have any significant effect on MRS catalytic activity (Figure 4E). From the various experiments, I could conclude that BC-MCI-CG-88 is the most promising drug candidate among all the test compounds.

6. The specific effect of BC-MCI-CG-88 on $p16^{INK4a}$-negative cancer cells

During the screening process, I only used H460 cell line as a $p16^{INK4a}$-negative cancer, therefore the effect of BC-MCI-CG-88 on CDK4 level was confirmed further using other cancer cell lines. A total of 7 cell lines were treated with BC-MCI-CG-88. BC-MCI-CG-88 noticeably reduces CDK4 level specifically in $p16^{INK4a}$-negative cancer cells and does not alter CDK7 and MRS (Figure 5A). In $p16^{INK4a}$-positive normal and cancer cells, BC-MCI-CG-88 did not change the level of CDK4, CDK7 and MRS (Figure 5B and 5C). Altogether, it suggests that BC-MCI-CG-88 reduces CDK4 in the absence of $p16^{INK4a}$ by inhibiting the stabilizing effect of MRS on CDK4 (Figure 6).

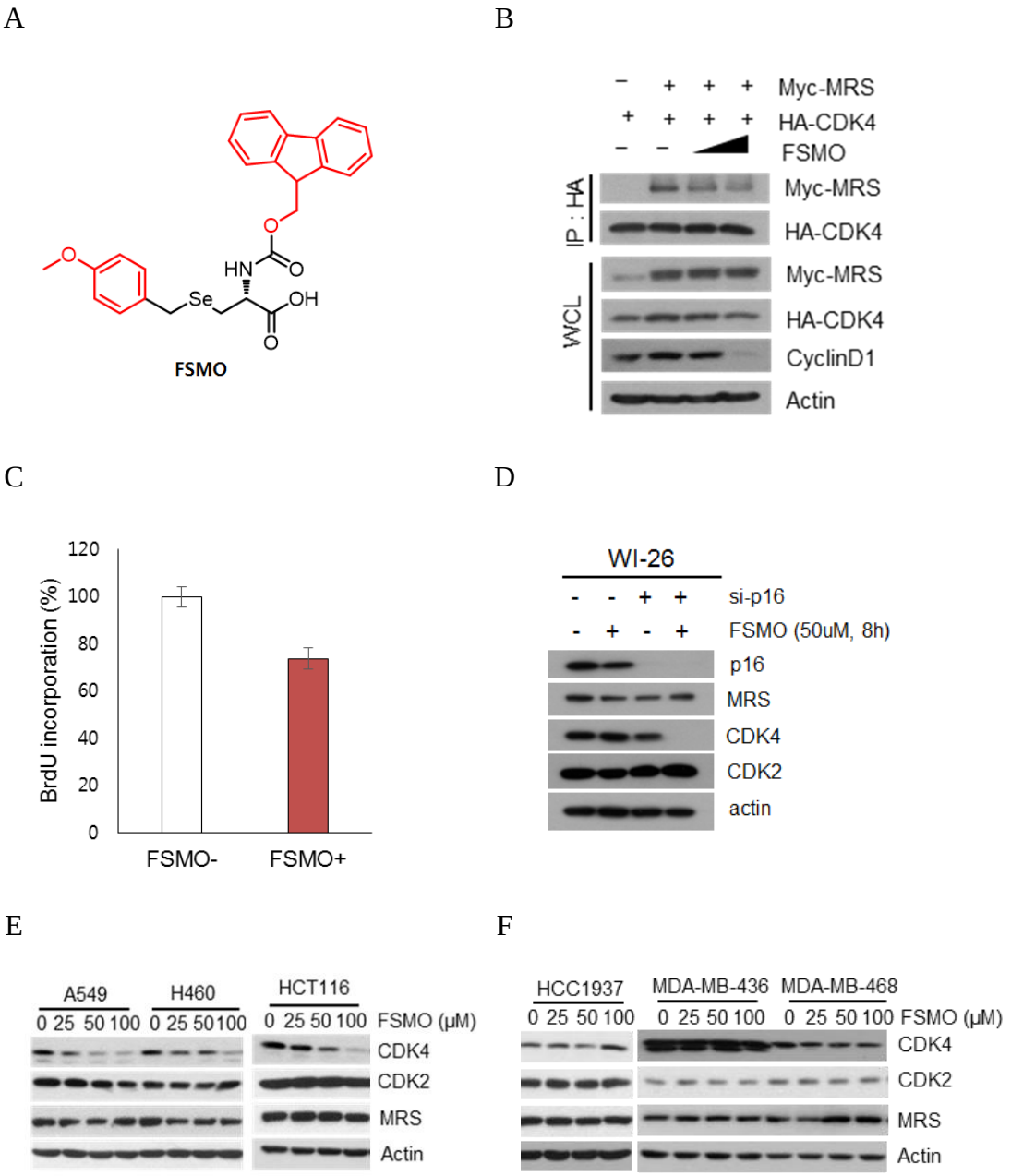
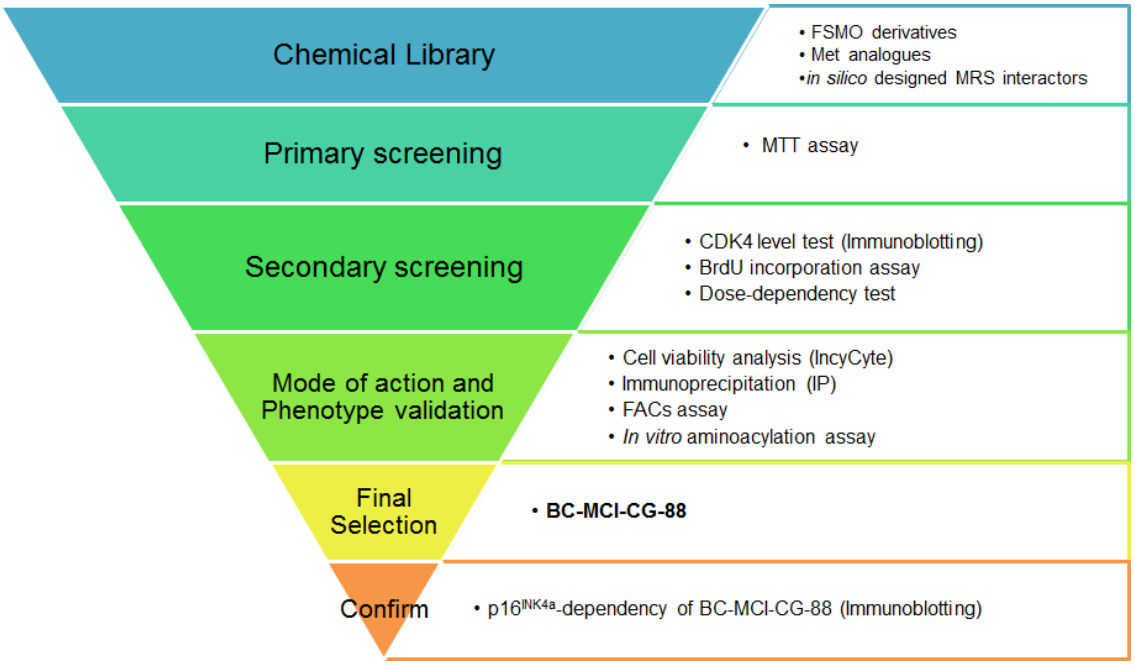


Figure 1. FSMO attenuates cell proliferation by interfering the MRS and CDK4 interaction on p16^{INK4a}-negative cancer

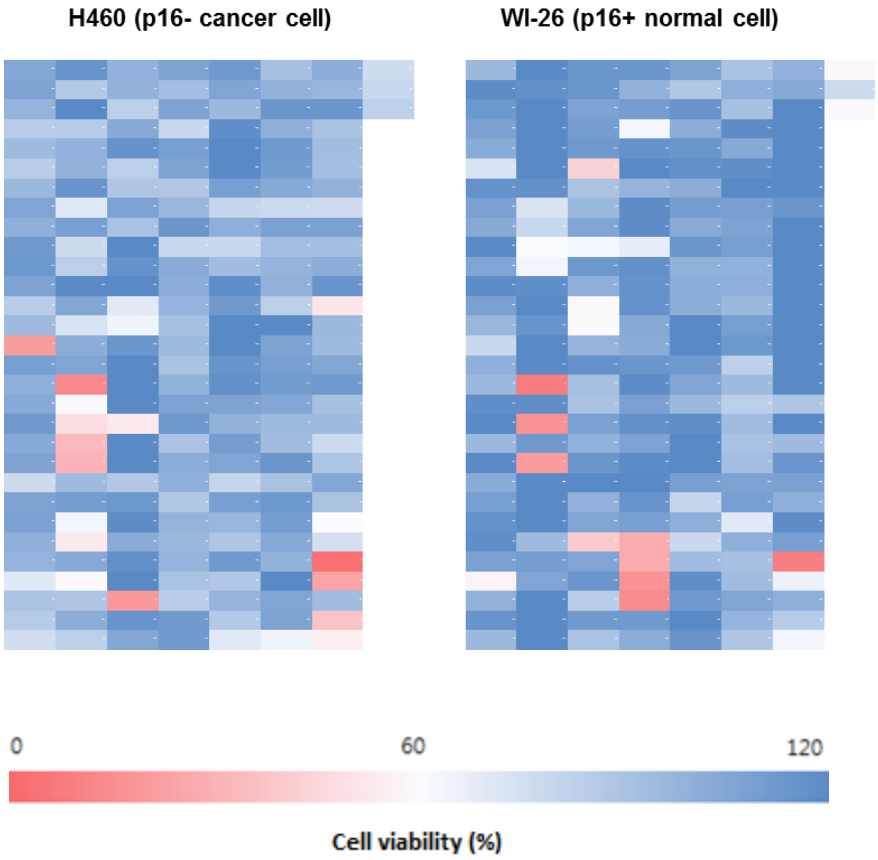
(A) Structure of Fmoc-Sec(Mob)-OH (FSMO) (B) H460 cells were transfected with Myc-MRS and HA-CDK4 and FSMO was treated for the concentration of 25 μM and 50 μM for 4 hours. The interaction between CDK4 and MRS was detected by immunoblotting after immunoprecipitation (IP). (C) MDA-MB-231 cells were treated with 50 μM FSMO for 8 hours. BrdU incorporation assay was used to detect the effect

on cell proliferation. FSMO treated cells showed less DNA synthesis (less BrdU incorporation during DNA replication) compare to DMSO-treated cells. (D) WI-26 cells, which are $p16^{INK4a}$-positive normal cells, were transfected si-Control or si-$p16^{INK4a}$ siRNA. After treated with 50 μM FSMO for 8 hours, cells were harvested and the effect of FSMO on CDK3 level was investigated. (E) $p16^{INK4a}$-negative cancer cells, and (F) $p16^{INK4a}$-positive cancer cells were treated with FSMO for 9 hours, then the level of CDK4 was investigated. CDK2 was included as a negative control.

A


Chemical Library
• FSMO derivatives
• Met analogues
• *in silico* designed MRS interactors
Primary screening
• MTT assay
Secondary screening
• CDK4 level test (Immunoblotting)
• BrdU incorporation assay
• Dose-dependency test
Mode of action and Phenotype validation
• Cell viability analysis (IncyCyte)
• Immunoprecipitation (IP)
• FACs assay
• *In vitro* aminoacylation assay
Final Selection
• **BC-MCI-CG-88**
Confirm
• $p16^{INK4a}$-dependency of BC-MCI-CG-88 (Immunoblotting)

B


H460 (p16- cancer cell)
WI-26 (p16+ normal cell)
0
60
120
Cell viability (%)

C

No.	Chemical	Type
1	BC-MCI-CG-50	*in silico* designed MRS interactor
2	BC-MCI-CG-55	*in silico* designed MRS interactor
3	BC-MCI-CG-57	*in silico* designed MRS interactor
4	BC-MCI-CG-79	*in silico* designed MRS interactor
5	BC-MCI-CG-88	*in silico* designed MRS interactor
6	BC-MCI-60	FSMO derivative
7	BC-MCI-76	FSMO derivative
8	BC-MCI-83	FSMO derivative

D

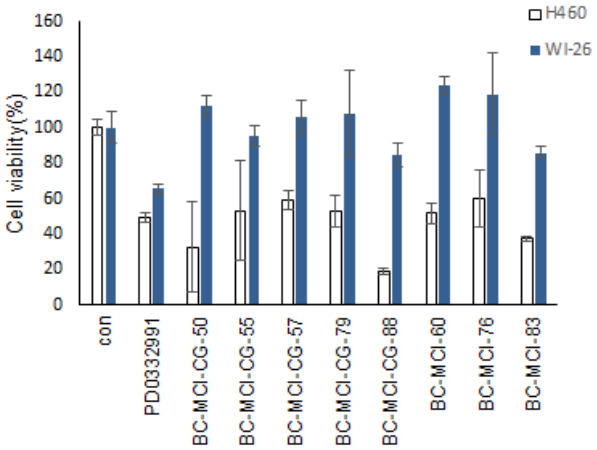
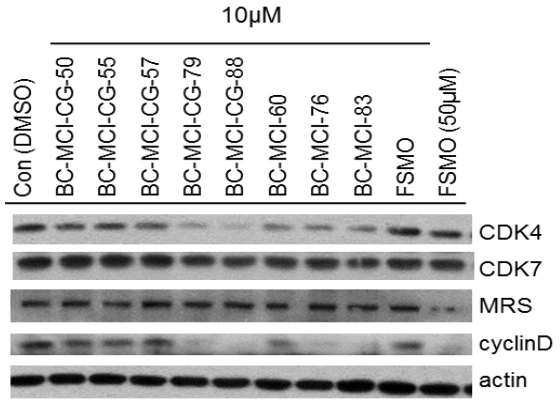


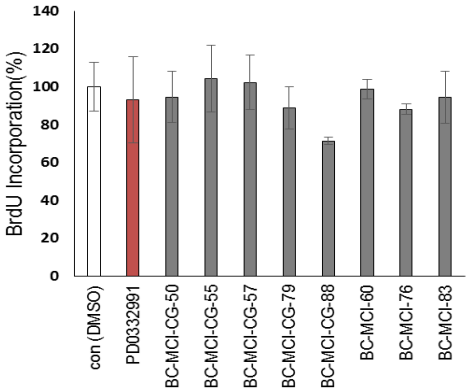
Figure 2. Screening strategy and heatmap for the effect of compounds on the proliferation of cancer and normal cells

(A) Screening flow to identify MRS-CDK4 interaction inhibitors. Primary and secondary assays were performed to narrow down the number of effective compounds and their effect on MRS activity was investigated. (B) Heat map of MTT assay data. A total of 213 chemicals were treated to H460 (p16INK4a-negative cancer cells), and WI-26 (p16INK4a-positive normal cells) for 72 hours at concentration of 10 μM. The relative cell viability (%) is presented in color (from blue to red). (C) A total of 8 chemicals were selected from the primary screening and (D) the MTT assay data is shown. The compounds were chosen based on less toxicity (cut-off: over 80% growth in WI-26 normal cells) and high efficacy (cut-off: under 60% growth in H460 cancer cells).

A



B



C

No.	Chemical	H460 IC_{50} (μM)	WI-26 IC_{50} (μM)
	PD0332991	8.67	6.32
1	BC-MCI-CG-88	3.99	19.45
2	BC-MCI-CG-83	6.89	20.44
3	BC-MCI-CG-50	7.99	NA
4	BC-MCI-CG-79	8.56	10.92
5	BC-MCI-60	11.79	24.39
6	BC-MCI-76	13.45	24.99
7	BC-MCI-CG-57	15.91	15.73
8	BC-MCI-CG-55	35.82	9.30

D

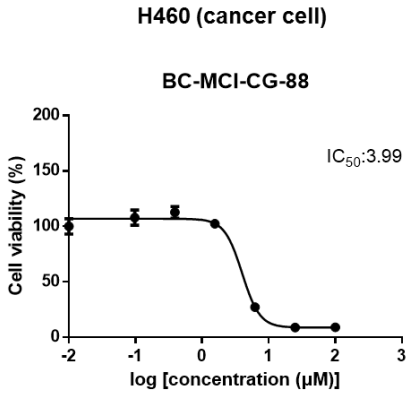
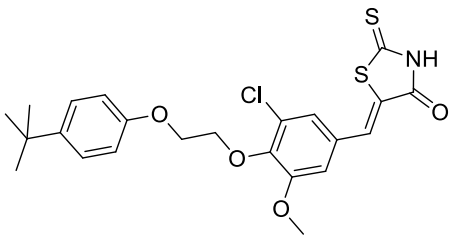


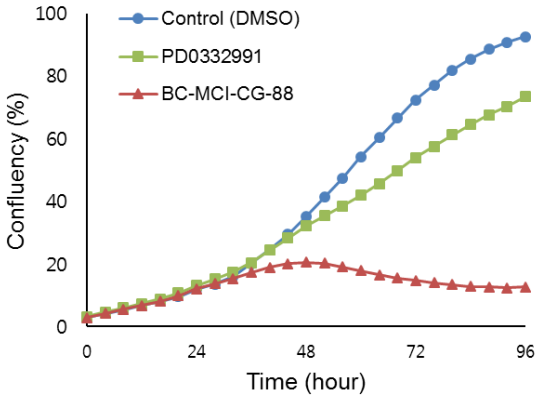
Figure 3. BC-MCI-CG-88 is the most potent compound, which inhibits cell proliferation by destabilizing CDK4

(A) H460 cells were treated with each compound (10 μM, except for FSMO at far right lane) for 6 hours and the CDK4 level was analyzed by immunoblotting using specific antibody. CDK2 was used as a negative control. (B) BrdU incorporation assay was performed to investigate the effect of each compound on cell proliferation. H460 cells were treated with each compound for 6 hours. (C) The half maximal inhibitory concentration (IC50) of each compound on the proliferation of H460 (lung cancer cell line) and WI-26 (normal cell line) were measured based on MTT assay. Cells were treated with each compound with the concentration range from 100 to 0.1 μM. (D) Dose-dependent effect of BC-MCI-CG-88 on the cell viability of H460.

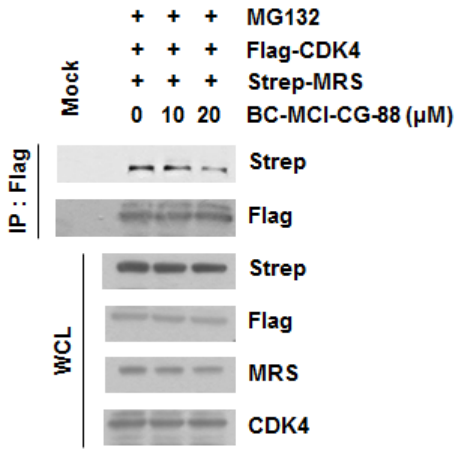
A


S
NH
S
O
Cl
O
O
O

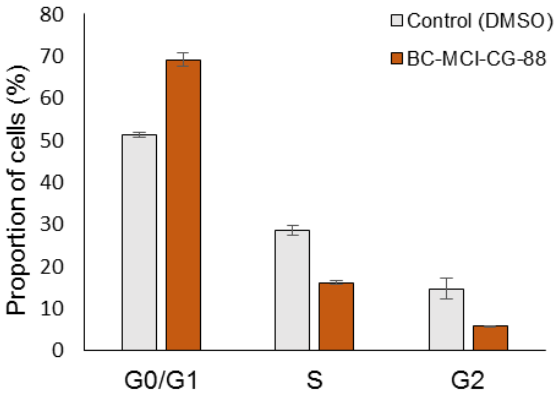
B


Control (DMSO)
PD0332991
BC-MCI-CG-88
Confluency (%)
100
80
60
40
20
0
0
24
48
72
96
Time (hour)

C


+ + + MG132
+ + + Flag-CDK4
+ + + Strep-MRS
0 10 20 BC-MCI-CG-88 (μM)
Mock
IP : Flag
Strep
Flag
WCL
Strep
Flag
MRS
CDK4

D


Control (DMSO)
BC-MCI-CG-88
Proportion of cells (%)
80
70
60
50
40
30
20
10
0
G0/G1
S
G2

E

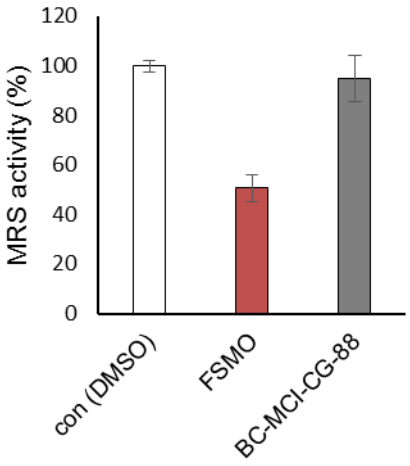

MRS activity (%)
120
100
80
60
40
20
0
con (DMSO)
FSMO
BC-MCI-CG-88

Figure 4. Characterization of BC-MCI-CG-88

(A) Structure of BC-MCI-CG-88 (B) H460 cells were treated with 10 μM BC-MCI-CG-88 (red triangle), and PD0332991 (green square) for 3 days and the real time cell proliferation was monitored with IncuCyte. (C) H460 cells were transfected with Flag-CDK4 and Strep-MRS and then treated with BC-MCI-CG-88. MG132 (Proteasome inhibitor) was pre-treated for 3 hours to inhibit proteasome-dependent CDK4 degradation. The effect of BC-MCI-CG-88 on the MRS and CDK4 interaction was investigated by immunoprecipitation. (D) Cell cycle analysis was performed using flow cytometry assay. H460 cells were treated with 10 μM BC-MCI-CG-88 for 6 hours. (E) To test the effect of BC-MIC-CG-88 on the catalytic activity of MRS, *in vitro* aminoacylation assay was done. FSMO was used as positive control.

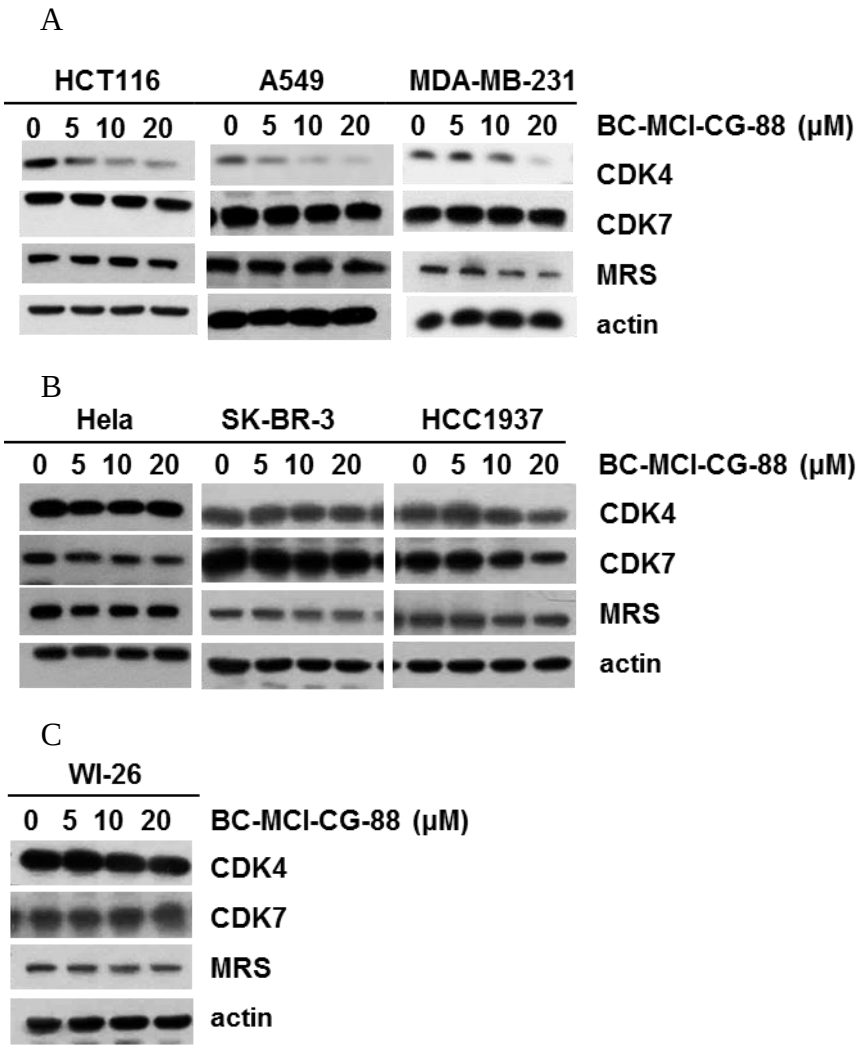


Figure 5. The effect of BC-MCI-CG-88 is dependent to the status of $p16^{INK4a}$

(A) $p16^{INK4a}$-negative cancer cells, (B) $p16^{INK4a}$-positive cancer cells, and (C) normal cells were treated with 10 μM of BC-MCI-CG-88 for 6 hours and the level change of CDK4 was determined by immunoblotting. CDK7 was used as a control.

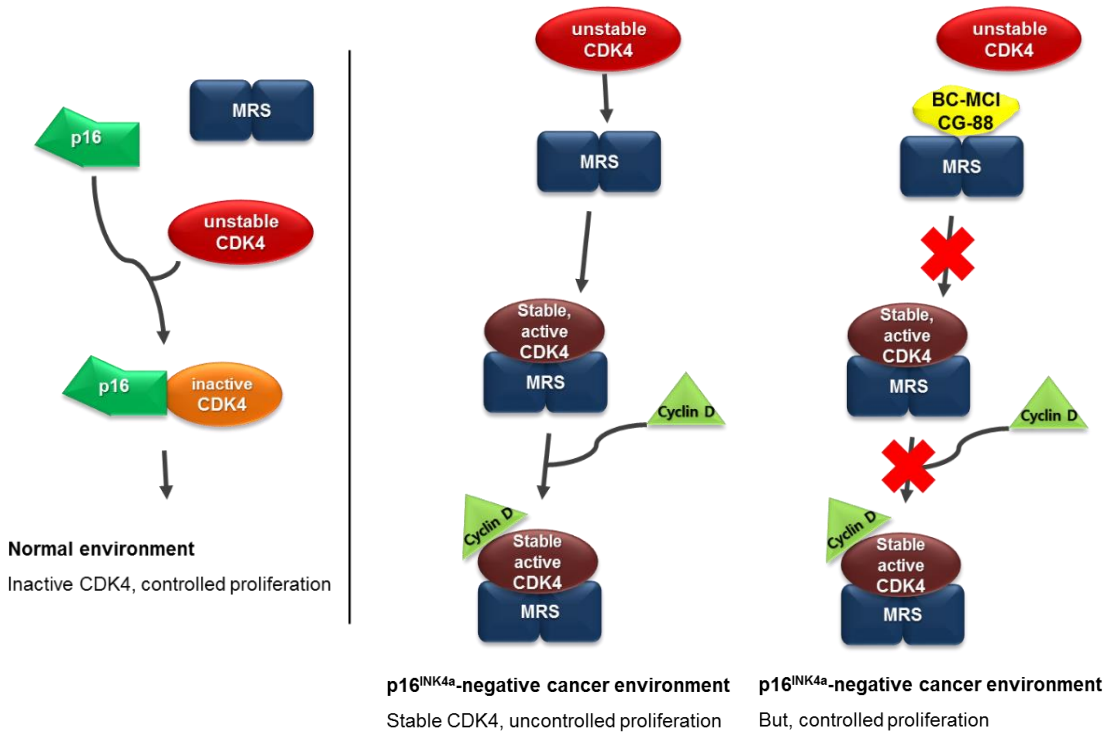


Figure 6. A schematic model for the function of BC-MCI-CG-88 on normal and p16^{INK4a}-negative cancer environment

In normal environment, p16^{INK4a} works as a tumor suppressor, which is responsible for cell-cycle regulation. When the control of proliferation is required, p16^{INK4a} binds to free CDK4 and inactivates it. In p16^{INK4a}-negative cancer environment, MRS takes places the binding site of p16^{INK4a} on CDK4, and stabilizes it resulting in uncontrolled proliferation. BC-MCI-CG-88 interferes the interaction between MRS and CDK4, destabilizes CDK4 and inhibits controlled proliferation in p16^{INK4a}-negative cancer.

Ⅳ. DISCUSSION

$p16^{INK4a}$ is a tumor suppressor and specific CDK4 inhibitor and its role in preventing cancer is well validated [7, 31]. Mouse model which had increased activity of ^{INK4a} and Arf protein, showed 3-fold less incidence of developing spontaneous tumorigenesis in the life span compare to normal mouse model [32]. In the same context, various kinds of mutations on tumor suppressor genes have been reported in cancers, and $p16^{INK4a}$ is one of the tumor suppressors which are frequently inactivated or mutated in cancers [5]. Loss of $p16^{INK4a}$ is closely related to the tumor development often used as a marker for predicting prognosis [8]. It is demonstrated from the COSMIC (Catalogue of Somatic Mutations in Cancer) database that $p16^{INK4a}$ inactivation is occurred in thousands of human cancers [33, 34], and for example, primary tumors with abnormal $p16^{INK4a}$ protein status are found up to 60% in pancreas, 40% in breast and 35% in lung [5, 7].

While $p16^{INK4a}$ is considered as a crucial factor for cancer development, there is no therapy specifically targeting the $p16^{INK4a}$-negative cancer patients. In this study, I demonstrated that MRS-CDK4 interaction inhibitor possess the possibility to become the first-line treatment for the patients devoid of $p16^{INK4a}$. BC-MCI-CG-88 compound specifically interfered the interaction of MRS and CDK4 and delivered cytotoxicity to $p16^{INK4a}$-negative cancer cells but not to normal cells.

Because FSMO was found only for the proof of concept in the previous study and it worked at two digits micromolar concentrations with pharmacophore not suitable for drug development, it was insufficient to become ‘Hit’ of drug discovery in many aspects. I focused on finding more ‘druggable’ chemical than FSMO and confirmed the mechanism and the effect of the chemicals. In this study, I did not use random library but used 3 different compound groups which were specifically designed for MRS

targeting. The compounds were FSMO derivatives, Met analogues and possible MRS interactors, which were guided by *in silico* modeling based on the non-human MRS structures. The composition of the library enabled me to do the primary screening based on phenotypic MTT assay, which is more effective for screening rather than MRS catalytic activity assay or MRS-CDK4 interaction assay. Finally, I could identify BC-MCI-CG-88, which is a possible MRS interactor with different pharmacophore with that of FSMO but interferes the binding between MRS and CDK4 like FSMO. BC-MCI-CG 88 compound has merits over FSMO in three reasons.

First, the result of MTT assay shows that this compound is effective on inhibiting cell proliferation of $p16^{INK4a}$ –negative cancer cells with only one-fifth of concentration of FSMO. Even in lower dose, BC-MCI-CG-88 reduces the level of CDK4 significantly. It was confirmed through immunoprecipitation assay that this effect is related to MRS-CDK4 interaction as it was previously observed with FSMO.

Secondly, BC-MCI-CG-88 did not changed the catalytic activity of MRS in higher dose, which means that it has low chance to have toxic effect on normal cells by interfering protein synthesis.

Finally, BC-MCI-CG-88 has better efficacy and less toxicity compared to PD0332991. PD0332991 is a potent CDK4/6 inhibitor and the clinical efficacy of PD0332991 on ER-positive and HER2-negative breast cancer has been well established [10]. Surprisingly, BC-MCI-CG-88 showed better cytostatic effect on H460 cancer cells compared to PD0332991 based on the BrdU incorporation and FACS assay in this study. Although PD0332991 is effective at nanomolar concentration in sensitive cell lines, there are still other types of breast cancer cells which show resistant to PD0332991 [35]. In this study, I used H460 lung cancer cells, but not breast cancer cells, and there was no report for the effectiveness of PD0332991 on H460 cell line. Probably, H460 would have some characteristics which enables it to be resistant to PD0332991. Considering

the response of H460, it is promising that there would be cell lines which are more sensitive to BC-MCI-CG-88 than PD0332991. It suggests the possibility of MRS-CDK4 interaction inhibitors to be considered as an alternative for the PD0332991-irresponsive patients in future.

Despite the advantages, there are still more challenges. Since the efficacy and toxicity *in vitro* is not always correlates with the result *in vivo*, animal model experiments must be conducted. Further optimization is also crucial to find the chemical with better pharmcokinetics and pharmocodynamics.

In conclusion, it is apparent that BC-MCI-CG-88 showed the potential of MRS-CDK4 interaction inhibitors which can be developed based on the strategy for cancer therapeutics to treat $p16^{INK4a}$-negative cancers. Although more experiments must be performed to prove the working mechanism of this compound and its *in vivo* efficacy, BC-MCI-CG-88 is meaningful in that it controls cell cycle of $p16^{INK4a}$-negative cancer by inhibiting MRS-CDK4 interaction without affecting catalytic activity of MRS. By optimizing this compound, a novel therapeutics expected to overcome the demerits of current CDK4/6 kinase inhibitors can be obtained.

Ⅴ. REFERENCE

1. Dickson, M.A., *Molecular Pathways: CDK4 inhibitors for cancer therapy.* Clinical Cancer Research, 2014. **20**(13): p. 3379-3383.
2. Sherr, C.J. and J.M. Roberts, *Inhibitors of mammalian G1 cyclin-dependent kinases.* Genes and Development, 1995. **9**(10): p. 1149-1163.
3. Zou, X., et al., *Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence.* Genes & development, 2002. **16**(22): p. 2923-2934.
4. Witkiewicz, A.K., et al., *The meaning of p16^{INK4a} expression in tumors: functional significance, clinical associations and future developments.* Cell Cycle, 2011. **10**(15): p. 2497-2503.
5. Liggett, W.H. and D. Sidransky, *Role of the p16 tumor suppressor gene in cancer.* Journal of Clinical Oncology, 1998. **16**(3): p. 1197-1206.
6. Young, R.J., et al., *Loss of CDKN2A expression is a frequent event in primary invasive melanoma and correlates with sensitivity to the CDK4/6 inhibitor PD0332991 in melanoma cell lines.* Pigment Cell & Melanoma Research, 2014. **27**(4): p. 12.
7. Serrano, M., et al., *Role of the ^{INK4a} locus in tumor suppression and cell mortality.* Cell, 1996. **85**(1): p. 27-37.
8. Straume, O., L. Sviland, and L.A. Akslen, *Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma.* Clinical Cancer Research, 2000. **6**(5): p. 1845-1853.
9. Mäkitie, A.A., et al., *Loss of p16 expression has prognostic significance in human nasopharyngeal carcinoma.* Clinical Cancer Research, 2003. **9**(6): p. 2177-2184.
10. Beaver, J.A., et al., *FDA Approval: Palbociclib for the treatment of postmenopausal*

Patients with estrogen receptor–positive, HER2-egative metastatic breast cancer. Clinical Cancer Research, 2015. **21**(21): p. 4760-4766.

11. Schwartz, G.K. and M. Dickson, *Development of cell cycle inhibitors for cancer therapy.* Current Oncology, 2009. **16**(2): p. 36-43.
12. Roberts, P.J., et al., *Multiple roles of cyclin-fependent kinase 4/6 inhibitors in cancer therapy.* Journal of the National Cancer Institute, 2012. **104**(6): p. 476-487.
13. Tetsu, O. and F. McCormick, *Proliferation of cancer cells despite CDK2 inhibition.* Cancer Cell, 2003. **3**(3): p. 233-245.
14. Ortega, S., et al., *Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice.* Nature Genetics, 2003. **35**(1).
15. Altenburg, J.D. and S.S. Farag, *The potential role of PD0332991 (Palbociclib) in the treatment of multiple myeloma.* Expert Opinion on Investigational Drugs, 2015. **24**(2): p. 261-271.
16. Finn, R.S., et al., *Abstract CT101: Final results of a randomized Phase II study of PD 0332991, a cyclin-dependent kinase (CDK)-4/6 inhibitor, in combination with letrozole vs letrozole alone for first-line treatment of ER+/HER2-advanced breast cancer (PALOMA-1; TRIO-18).* Cancer Research, 2014. **74**(19 Supplement): p. CT101-CT101.
17. UNC Lineberger Comprehensive Cancer Center. *Phase II trial of Palbociclib in patients with metastatic urothelial cancer after failure of first-line chemotherapy*. 2016 Available from: https://clinicaltrials.gov/ct2/show/NCT02334527?term=palbociclib+trial&rank=2.
18. National Cancer Institute. *Ibrutinib and Palbociclib in treating patients with previously treated mantle cell lymphoma*. 2016; Available from: https://clinicaltrials.gov/ct2/show/NCT02159755?term=pd0332991+trial&rank=10.
19. Pollak, A., *Guarded optimism after breast cancer drug shows promising results*, in

The New York Times. 2014.

20. Beasley, D., *Pfizer drug doubles time to breast cancer tumor growth in trial*, in *Reuters*. 2014.
21. Paternot, S., et al., *The CDK4/CDK6 inhibitor PD0332991 paradoxically stabilizes activated cyclin D3-CDK4/6 complexes.* Cell Cycle, 2014. **13**(18): p. 2879-2888.
22. Cordon-Cardo, C., *Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia.* The American journal of pathology, 1995. **147**(3): p. 545.
23. Krystof, V. and S. Uldrijan, *Cyclin-dependent kinase inhibitors as anticancer drugs.* Current Drug Targets, 2010. **11**(3): p. 291-302.
24. Berthet, C. and P. Kaldis, *Cell-specific responses to loss of cyclin-dependent kinases.* Oncogene, 2007. **26**(31): p. 4469-4477.
25. Malumbres, M. and M. Barbacid, *Cell cycle, CDKs and cancer: a changing paradigm.* Nature Reviews Cancer, 2009. **9**(3): p. 153-166.
26. Reddy, H.K., et al., *Cyclin-dependent kinase 4 expression is essential for neu-induced breast tumorigenesis.* Cancer Research, 2005. **65**(22): p. 10174-10178.
27. Yu, Q., et al., *Requirement for CDK4 kinase function in breast cancer.* Cancer Cell, 2006. **9**(1): p. 23-32.
28. Kim, S. and T.J. Bullwinkle, *Aminoacyl-tRNA synthetases in biology and medicine*, in *Topics in Current Chemistry,*. 2014, Springer,: Dordrecht. p. 1 online resource (x, 350 pages).
29. Kwon, N.H., *Methionyl-tRNA synthetase promotes cell cycle via cyclin-dependent kinase 4.* to be published.
30. Alao, J.P., *The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention.* Molecular Cancer, 2007. **6**(1): p. 1.
31. Esteller, M., et al., *Detection of aberrant promoter hypermethylation of tumor*

suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Research, 1999. **59**(1): p. 67-70.

32. Matheu, A., et al., *Increased gene dosage of INK4a/Arf results in cancer resistance and normal aging.* Genes & development, 2004. **18**(22): p. 2736-2746.
33. Forbes, S., et al., *COSMIC 2005.* British Journal of Cancer, 2006. **94**(2): p. 318-322.
34. Kim, W.Y. and N.E. Sharpless, *The regulation of INK4/ARF in cancer and aging.* Cell, 2006. **127**(2): p. 265-275.
35. Finn, R.S., et al., *PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro.* Breast Cancer Research: BCR, 2009. **11**(5): p. R77.

요약 (국문초록)

Methionyl-tRNA synthetase와 cyclin-dependent kinase 4 결합의 화학적 억제와 $p16^{INK4a}$ 음성 암세포에 대한 효과

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약학과 의약생명과학전공

Aminoacyl-tRNA synthetases (ARSs)는 transfer RNA (tRNA)에 상보적인 아미노산을 붙여주는 효소로 단백질 합성에서 중요한 역할을 담당한다. 또한 ARSs는 다기능 단백질로 다양한 암 세포의 신호전달에 관여, 세포 생존 및 사멸, 전이, 면역 등에서 중요한 역할을 수행한다. 이중 methionyl-tRNA synthetase (MRS)는 단백질 번역의 개시에 필수적인 아미노산인 메티오닌 (Methionine)을 담당한다. 자외선 등의 스트레스 상황에서는 인산화되어 단백질 합성을 억제하고 핵산 손상 제거 기전을 활성화 시키는 등 스트레스에 반응하여 세포질의 단백질 합성 과정을 핵산 조절 과정과 연관시켜 적절한 세포 반응을 유도한다. 최근 선행연구를 통해 MRS가 Cyclin-dependent kinase 4 (CDK4)와 결합해 CDK4 안정화에 기여한다는 사실이 밝혀졌다. MRS의 CDK4 조절효과는 CDK4 억제제인 $p16^{INK4a}$ 결여 암세포에서 명확히 관찰되었다. CDK4는 여러 암 종, 특히 유방암의 개시 및 유지에 필수적인 것으로 알려져 있으며 최근 CDK4/6 억제제인 PD0332991이 FDA 승인을 받아 그 관심과 중요성이 증대되고 있는 검증된 암 치료 타겟 단백질이다. 하지만 PD0332991의 사용은 예상치 않게 cyclin D3-CDK4/6 복합체 안정화를 유도한다는 보고가 있으며 다른 인산화효소 억제제에서 보여지듯이 CDK4/6 돌연변이에 의한 내성 출현이 예상되고 있다. 따라서 PD0332991과는 다른 기전을 갖는 CDK4 조절 치료제의 개발은 중요한 의미를

갖는다.

우리는 선행연구를 통해 메티오닌 유도체인 Fmoc-Sec(Mob)-OH (FSMO)가 MRS와 CDK4사이의 결합을 방해하고 세포성장을 줄인다는 사실을 밝혀내었다. 하지만, FSMO는 신약개발에 적합한 약물분자구조가 아니고 상대적으로 높은 농도에서 작동하며 MRS 고유 활성에도 영향을 주어 정상세포에서 독성을 나타낼 우려가 있다. 이러한 단점들을 극복한 새로운 화합물을 발굴하기 위해 우리는 약 200여종의 화합물을 이용한 연구를 수행하였다.

암 세포 및 정상 세포를 활용한 세포증식 효과 분석을 통해, FSMO에 우수한 특이성과 암세포 사멸효과를 보이는 BC-MCI-CG-88 화합물을 발굴하였다. BC-MCI-CG-88은 특히 $p16^{INK4a}$ 음성 암세포에서 효과를 보이며 MRS와 CDK4의 결합을 방해함으로써 CDK4의 안정화를 막는다. 또한 이 화합물은 MRS의 활성에는 영향을 미치지 않으면서 세포주기를 조절함을 세포주기 분석 및 MRS 활성 측정기법을 통해 밝혀내었다. 결과적으로, BC-MCI-CG-88은 MRS-CDK4 결합에 의한 CDK4 안정화를 막아 암 세포 주기 및 암세포 성장을 억제한다. 특히 이 효과는 $p16^{INK4a}$ 가 변이 혹은 결여된 암세포에서 나타나므로 정상세포에 미치는 독성 효과가 적을 것으로 예상된다. 본 연구는 $p16^{INK4a}$-음성 암의 새로운 항암요법제로서 MRS 저해제의 활용 가능성을 밝혔다는 데 의의가 있다.

주요어 : MRS (Methionyl-tRNA synthetase), CDK4 (cyclin-dependent kinase 4), $p16^{INK4a}$ (cyclin-dependent kinase inhibitor 2A), 메티오닌 유도체, 세포주기 억제제

학번 : 2014-25280