이학석사학위논문

A deguelin analogue, SH48 inhibits STAT3 signaling in breast cancer cells

인체유방암세포에서 STAT3 signaling을 억제하는 deguelin 유도체 SH48에 대한 연구

2014년 2월

서울대학교 대학원

분자의학및바이오제약학과

홍성준

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이 논문을 이학석사학위논문으로 제출함

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홍성준의 석사학위논문을 인준함

2014년 2월

위 원 장 _____(인)

부위원장 _____(인)

위 원 _____(인)

Abstract

A deguelin analogue, SH48 inhibits STAT3 signaling in breast cancer cells

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Constitutively activated signal transducer and activator of transcription 3 (STAT3) plays an important role in oncogenesis and tumor development. Depending on the cell type and stimulus, STAT3 mediates different anti-proliferative events effects, such as apoptosis, autophagy and senescence. STAT3 is activated through tyrosine phosphorylation, which leads to its dimerization and translocation to the nucleus. In the present study, a new STAT3 inhibitor, SH48 discovered by virtual screening, was found to inhibit phosphorylation, nuclear translocation and transcriptional activity of STAT3 in human mammary epithelial MCF-10A cells transfected with *ras* oncogene (MCF10A-*ras*). SH48 has an α,β-unsaturated carbonyl group in its structure, and is speculated to interact with a thiol residue of STAT3, thereby

inactivating this transcription factor. The thiol-reducing agents, dithiothreitol or N-acetylcysteine abolished the SH48-induced suppression of STAT3 activation. In contrast to SH48, non-electrophilic analogues of SH48 failed to inhibit STAT3 activation. By utilizing a biotinylated SH48, we were able to demonstrate the interaction between SH48 and STAT3. SH48 binds to STAT3 directly via Michael addition, and thereby inhibits STAT3 activation. SH48 may also make hydrogen bonding with STAT3, but this interaction is not likely to inhibit STAT3 phosphorylation. SH48 induces cell death in MCF10A-*ras* cells, but not in normal MCF10A cells. SH48-induced cell death was not associated with apoptosis, but it upregulated the expression of LC3Ⅱ and p62 involved in autophagy. SH48 does induce autophagic cell death (type II cell death) as determined by FACS analysis of Lysotracker fluorescence in MCF10A-*ras* cells. Autophagy induced by SH48 resulted in suppression of tumor growth, which appears to be dependent on its ability to inhibit STAT3 activation. In conclusion, SH48 induces autophagy by directly targeting STAT3.

Keywords: STAT3, SH48, α, β-unsaturated carbonyl group, Michael addition, Autophagy

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Contents

List of Figures

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Introduction

Deguelin is a natural product from the African plant, *Mundulea sericea*, and well known to regulate NFκB a transcription factor overactivated in many cancerous and transformed cells [1]. Deguelin has a chemopreventive and a therapeutic effect against lung cancer [2]. In addition, deguelin inhibits the AKT pathway and induces apoptosis in breast cancer cells [3]. Signal transducer and activator of transcription (STAT) family proteins play important roles in cell growth and development [4]. Among these, STAT3 is a transcription factor that is involved in oncogenesis and tumor development [5, 6]. STAT3 is constitutively activated in several cancer cells [7].

STAT3 phosphorylation at tyrosine residue 705 facilitates STAT3 dimer formation, which leads to nuclear translocation of STAT3 and its binding to a specific DNA site, thereby inducing target gene transcription [5]. The STAT dimer is formed via the reciprocal interaction between the SH2 domain of one monomer and the phosphorylated tyrosine of the other. [8]. The STAT3 dimer is translocated in the nucleus and recognizes a specific DNA binding site [9]. Tyrosine dephosphorylation of STAT makes STAT relocate to the cytoplasm [10]. The dominant-negative STAT3 suppresses *src* transformation and slows the growth of the cell lines developed from head and neck cancers [9, 11, 12].

Thus, inhibition of STAT3 phosphorylation can reduce STAT3 activation, and suppresses oncogenesis.

It has been reported that STAT3 represses autophagy in human cells, while inhibition of STAT3 can cause autophagy [13]. Inhibitors of STAT3 upregulate the autophagic flux [14]. Autophagy is a self-eating mechanism. In autophagy, phagophore engulfs intracellular organelles or aberrant proteins and then makes autophagosome with LC3Ⅱ and p62. Autophagosome fused with lysosomes becomes autolysosome [13, 15]. Autolysosome is eventually degraded and it supplies nutrition to the surrounding environment.

We synthesized many analogues of deguelin. It is known that the α,β-unsaturated carbonyl group is required for inhibition of STAT3 phosphorylation and NF-κB activation [16].

We have synthesized a novel structural analogue SH48 that has an α,β-unsaturated carbonyl moiety. Here, we report that SH48 can induce cell proliferation and induce autophagy in MCF10A-*ras* cells. However, SH48 was barely cytotoxic to normal MCF10A cells, so hence has a potential to be developed as an anticancer drug.

Materials and Methods

Chemicals

SH48, SH37, SH39, SH58, SH57, SH60, SH61, SH56, SH55, SH42, SH43, SSI1204 and SSI1205 were obtained from Prof. Young-Ger Suh`s lab (College of Pharmacy, Seoul National University). Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and horse serum were obtained from Gibco BRL (Grand Island, NY, USA). Cholera toxin, hydrocortisone, insulin, human epidermal growth factor (h-EGF) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). pSTAT3-TA-Luc plasmid was purchased from BD Biosciences Clontech (Palo. Alto, CA). Antibodies against pSTAT3, STAT3, LC3Ⅱ, p62, Bcl-x_L, CyclinD1, HA-tag and Myc-tag were obtained from Cell Signaling (Cell Signaling Technology, Beverly, MA, USA). Antibodies against BECN1 and agarose immunoprecipitation reagent were products of Santa Cruz Biotechnolgies Co. (Santa Cruz, CA, USA). Secondary antibodies, STAT3 short interfering RNA (siRNA), negative control siRNA, and lipofectamin RNAi-MAX reagent were purchased from Invitrogen Life Technologies, Inc.

(Carlsbad, CA, USA). All other chemicals used were of analytical or the highest purity grade available.

Cell culture

MCF10A and MCF10A-*ras* cells were cultured in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum, 10 μg/mL insulin, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone, 20 ng/mL h-EGF, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin. MCF7 and PC3 cell lines were grown in RPMI medium supplemented with 10% (v/v) heat-inactivated FBS (Biowhittaker Inc., Walkerville, MD), 100 U/ml penicillin and 100 g/ml streptomycin. HeLa/STAT3-luc and MDA-MB-231 cells were maintained in DMEM supplemented with 10% fetal bovine serum and an 100 ng/ml penicillin/streptomycin/fungizone mixture. These cell lines were grown at 37ºC in a humidified air/CO_2 (19:1) atmosphere. The cells were plated at an appropriate density according to each experimental scale.

MTT reduction assay

Cells were plated at a density of 2.5 x 10^4 cells/200 μl in 48-well plates, and the cell viability was determined by the MTT reduction assay. After incubation, cells were treated with the MTT solution (final concentration, 1 mg/ml) for 2 h. The dark blue formazan crystals formed in intact cells were

dissolved with dimethyl sulfoxide (DMSO) and the absorbance at 570 nm was read using a microplate reader. Results were expressed as the percentage of MTT reduction obtained in the treated cells, assuming that the absorbance of control cells was 100%.

Transient transfection and the luciferase reporter assay

HeLa/STAT3-luc cells were seeded at a density of 2 x 10^5 per well in a six-well dish and grown to 60% to 80% confluence in the complete growth medium. Cells were pretreated with test compounds for 24 h, stimulated with 10 ng/mL oncostatin M for 5 h. The cells were then washed with PBS and lysed in 1x reporter lysis buffer (Promega). The lysed cell extract (20 μL) was mixed with 100 μL of the luciferase assay reagent, and the luciferase activity was determined using a luminometer (AutoLumat LB 953, EG&G Berthold). The β-galactosidase activity was measured to normalize the luciferase activity.

siRNA knockdown of STAT3.

The human STAT3 siRNA duplex and negative control siRNA were purchased from KDR, Inc. The sequences of each siRNA were as follows: STAT3 siRNA-1 (sense, 5"-UGUUCUCUGAGACCCAUGAdTdT-3"; antisense, 5"-UCAUGGGUCUCAGAGAACAdTdT-3")

Western blot analysis

MCF10A-*ras* cells were lysed in lysis buffer [250 mmol/L sucrose, 50 mmol/L Tris-HCl (pH 8.0), 25 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 2 mmol/L NaF, 2 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonylfluoride] for 15 min on ice followed by centrifugation at 13,000 g for 15 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockfold, IL, USA). Protein (30 μg) was separated by running through 8% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% nonfat dry milk PBST buffer (PBS containing 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilutionof one of the antibodies of pSTAT3, STAT3, PARP, SQSTM1/p62, CyclinD1, Bcl-x_L, LC3B (Cell Signaling Technology, Beverly, MA, USA) or BECN1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal lane loading was assured using actin (Sigma Chemical Co., St. Louis, MO). The blots were rinsed three times with PBST buffer for 10 min each. Washed blots were treated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Pierce Biotechnology, Rockford, IL, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunoprecipitation

MCF10A-*ras* cells were treated with biotinylated SH48 for 24 h, and cells were lysed in 250 mmol/L sucrose, 50 mmol/L Tris-HCl (pH 8.0), 25 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 2 mmol/L NaF, 2mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonylfluoride. Total protein (500 μg) was subjected to immunoprecipitation by shaking with STAT3 primary antibody at 4°C for 12 h followed by the addition of protein A/G-agarose bead suspension (25% slurry, 20 mL) and additional shaking for 2 h at 4°C. After centrifugation at 10,000 rpm for 1 minute, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 8μL of 6x SDS electrophoresis sample buffer and boiled for 5 minutes. Supernatant (48 μL) from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel. The incorporation of biotinylated SH48 into immunoprecipitated proteins was visualized by use of Amersham streptavidin–horseradish peroxidase (HRP) conjugate (GE Healthcare).

Immunocytochemistry

Transfected PC3 cells were plated on the chamber slide at a density of 5 x 10^4 cells/ml and treated with SH48 or DMSO. Cells were fixed with 95% methanol/5% acetic acid at -20℃ for 5 min, washed with PBS twice, treated with 0.2% Triton X-100 in PBS for 5 min, and washed with PBST then with

PBS. Samples were incubated with blocking agent [0.1% Tween-20 in PBS containing 5% bovine serum albumin (BSA)] at room temperature for 1 h, washed with PBS, then incubated with diluted (1:100) primary antibody for overnight at 4℃. After washing with PBS, samples were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG secondary antibody in PBST containing 1% BSA at room temperature for 1 h. Samples were washed with 0.1% PBST containing 1% BSA then examined under a fluorescent microscope.

Statistical analysis

When necessary, data were expressed as means ± SDs of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t-test*. The criterion for statistical significance was $P < 0.05$.

Results

Discovery of SH48 and comparative effect of analogues on cell viability

Based on the deguelin structure, several analogues with a modified deguelin skeletal structure were prepared **(Fig. 1A)**. To find out which compound is most effective for suppressing STAT3, the STAT3 transcriptional activity of analogues were compared by the luciferase gene reporter assay. The HeLa/STAT3-luc cell line was treated for 24 h and stimulated with oncostatin M (10 ng/ml) for 5 h. As shown here, SH48 was found to be the most potent inhibitor of STAT3 **(Fig. 1B)**.

SH48 reduces cell viability in MCF10A-*ras* cells but not in MCF10A normal cells. STAT3 is constitutively expressed in MCF10A-*ras* cells but not in MCF10A normal cells. Some deguelin analogues also reduced the viability of MCF10A-*ras* cells. Again, SH48 is was most effective in terms of suppression of MCF10A-*ras* cell viability **(Fig 2A and 2B)**. MCF7 and MCF10A cells express pSTAT3 low, whereas MDA-MB-231 and MCF10A-*ras* cells have a high level of pSTAT3 [17]. MCF7 and MDA-MB-231 cells were treated with indicated concentrations of SH48 for 24 h and their viability was compared **(Fig. 2C)**. SH48 exerted cytotoxic effects in both of the cells, but MCF7 cells were less susceptible to SH48-induced cytotoxicity. Therefore,

SH48 appears to be more effective in STAT3-overexpressing cells, such as MDA-MB-231 and MCF10A-*ras* in exerting cytotoxic effects.

SH48 induces autophagy

The suppression of STAT3 induces apoptosis [18]. We determined whether a cytotoxic dose of SH48 could induce apoptosis of MCF-10A-*ras* cells overexpressing STAT3. However, we were unable to detect hallmarks of apoptosis, such as cleavage of PARP and reduction in the levels of the anti-apoptotic protein Bcl-x_L. For this reason, SH48-induced cell death is unlikely to be mediated by apoptosis **(Fig. 3)**.

Autophagy is a self-eating mechanism whereby cells digest itself during starvation. During autophagy, intracellular phagophore engulfs misfolded proteins of cellular organelles and then forms autophagosome. Autophagic vesicles and their contents are destroyed by the fusion of lysosomes. The role of autophagy in cancer is complex. Autophagic vesicles reflect the existence of type Ⅱ cell death, or instead adaptive response to maintain continual cell survival under stress condition [19].

It is known that STAT3 can interfere with autophagic pathways and that suppression of STAT3 induces autophagy [20]. siRNA knockdown of STAT3 did not show any increases in the level of the autophagic marker LC3Ⅱ (**Fig**

4).

LysoTracker Red is a fluorescent acidotropic probe used for labeling and tracking acidic organelles in live cells. MCF10A-*ras* cells were pretreated with SH48 and exposed to 100 nM of LysoTracker Red for 30 min. As shown in **Fig. 5**, SH48 induced autophagic cell death and upregulates autophagic marker p62 and LC3Ⅱ.

SH48 inhibits STAT3 dimerization and subsequentlyits nuclear translocation

As described above, SH48 suppresses the transcriptional activity of STAT3. In addition, SH48 reduced the level of phospho-STAT3 at the concentration which inhibited the STAT3 transcriptional activity (**Fig 6A**). The result of immunocytochemistry shows that SH48 suppresses nuclear translocation of STAT3 **(Fig 6B)**. pSTAT3^{Y705} stained with FITC-conjugated anti-rabbit IgG antibody was visualized as green fluorescence. The nuclear accumulation of pSTAT3 was lower in SH48 treated cells than that in the vehicle control.

STAT3 is tagged with HA and MYC. HA-tagged STAT3 is detected by green fluorescence and MYC tagged STAT3 by red fluorescence. The nuclear marker 4',6-diamidino-2-phenylindole (DAPI) is shown in blue fluorescence.

The combination of green, red and blue generates white. In DMSO, treated control cells, white spots are observed. In SH48 treated cells, the bright blue color of nucleus is seen while green or red one is less intense. These findings suggest that SH48 inhibits STAT3 dimerization and translocation into nucleus (**Fig. 7**).

The α,β-unsaturated carbonyl group of SH48 is critical in its suppression of STAT3 signaling in MCF10A-ras cells.

To eleucidate the mechanism by which SH48 inhibits STAT3, dithiothreitol (DTT) and N-acetylcysteine (NAC) were treated to MCF10A-*ras* cells together with SH48. These thiol-reducing agents abolished the SH48-induced suppression of STAT3 activation **(Fig 8A** and **8B)**. This obersvation suggest the direct interaction between SH48 and a cysteine residue present in STAT3.

SH48 inhibits STAT3 activation and upregulates autophagic marker p62. In contrast to SH48 bearing the α,β-unsaturated carbonyl moiety and hence capable of directly binding to the STAT3, the non-electrophilic analogues SH42 and SH43 that lacks such an electrophilic moiety failed to suppress STAT3 phosphorylation and upregulate p62 **(Fig 9)**.

In another experiment, the viability of MCF10A-*ras* cells treated with SH48, SSI1204 and SSI1205 was compared. SSI1204 has an α,β-unsaturated

carbonyl group but SSI1205 doesn`t have it. SSI1204 and SH48 reduced the cell viability but SSI1205 didn't. Thus, the α,β-unsaturated carbonyl group plays important role in induction of cell death through STAT3 inhibition **(Fig 10)**.

SH48 binds directly to STAT3.

To demonstrate the direct interaction between STAT3 and SH48, a biotinylated SH48 was used. The binding of biotin-conjugated SH48 to STAT3 was detected with HRP-streptavidin **(Fig 11)**. Treatment with the thiol reducing agent DTT abolished the interaction between SH48 and STAT3, suggesting the possible modification of STAT3 cystein thiol by SH48.

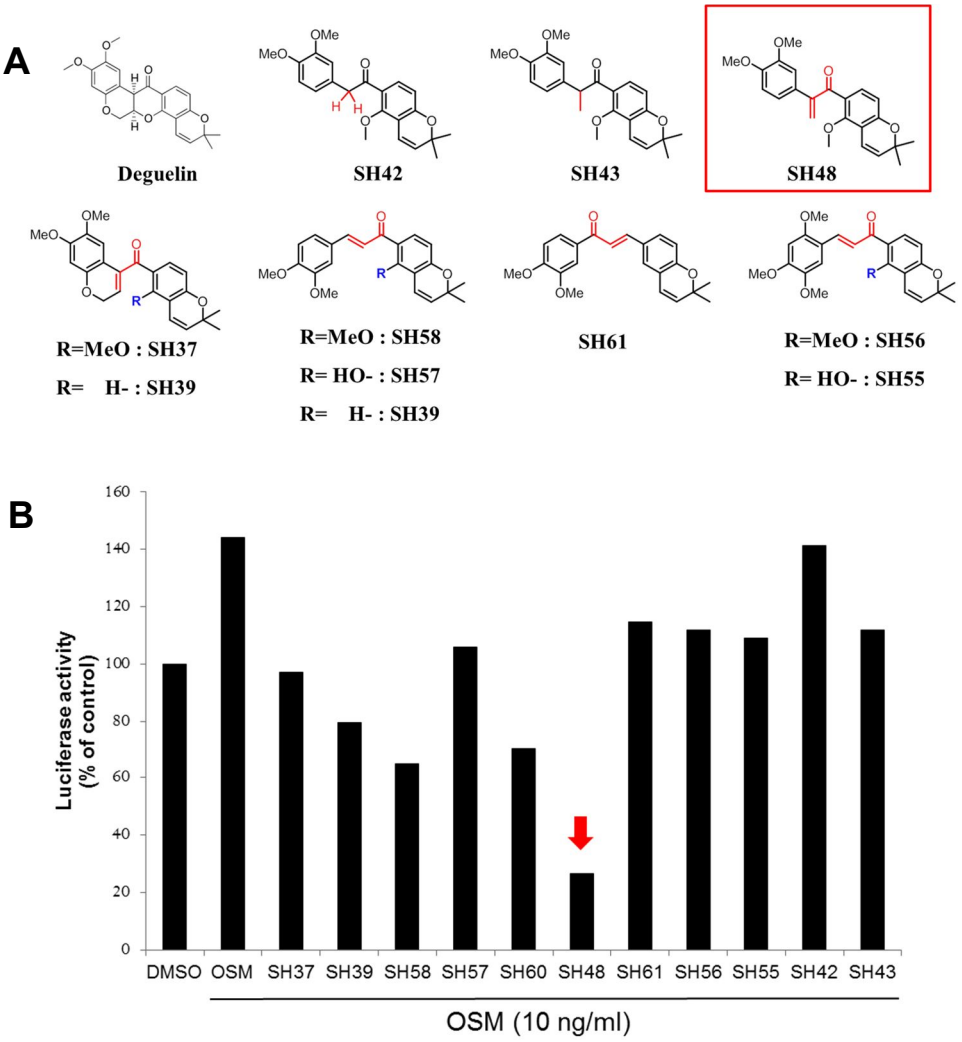
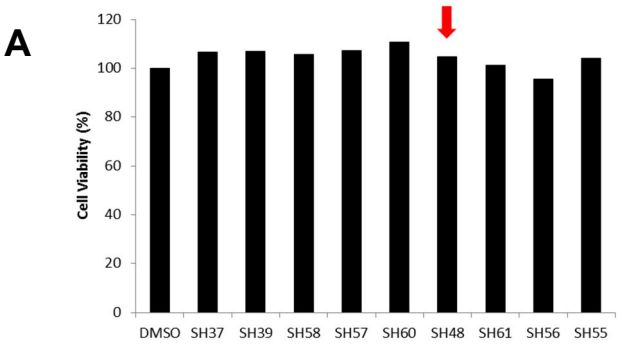
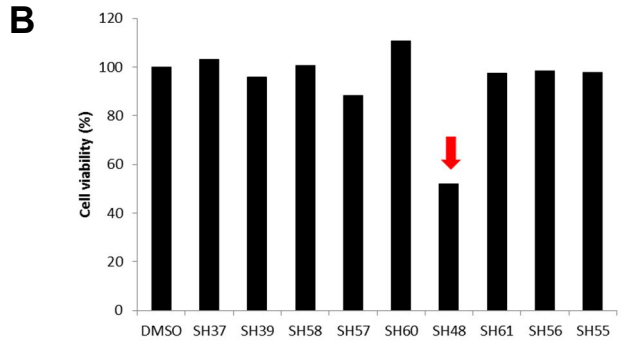


Fig. 1. Chemical structures of deguelin and its analogues and their effects on STAT3 transcription activity. (A) Chemical structures of deguelin and its analogues. (B) HeLa/STAT3-luc cells were pretreated with DMSO or each of analogues for 24 h, stimulated with oncostatin M (OSM) for 5 h, and then assayed for the luciferase reporter gene activity.


A
120
100
80
60
40
20
0
Cell Viability (%)
DMSO SH37 SH39 SH58 SH57 SH60 SH48 SH61 SH56 SH55


B
120
100
80
60
40
20
0
Cell viability (%)
DMSO SH37 SH39 SH58 SH57 SH60 SH48 SH61 SH56 SH55

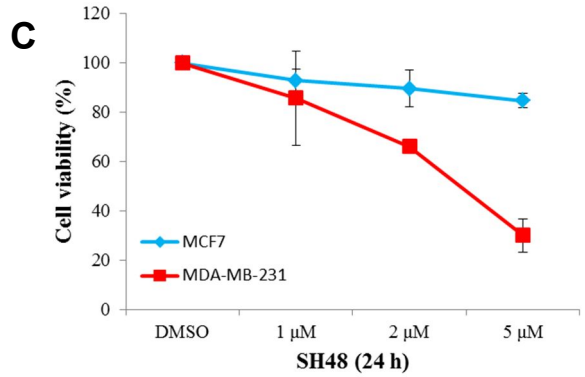

C
120
100
80
60
40
20
0
Cell viability (%)
MCF7
MDA-MB-231
DMSO
1 µM
2 µM
5 µM
SH48 (24 h)

Fig. 2. Comparative effects of deor guelin analogues on viability of normal MCF10A, MCF10A-*ras*, MCF7 and MDA-MB-231 cells. MCF10A (A) or MCF10A-*ras* (B) cells were treated with each of deguelin analogues for 24 h. (C) MCF7 and MDA-MB-231 cells were treated with indicated concentrations of SH48 for 24 h.

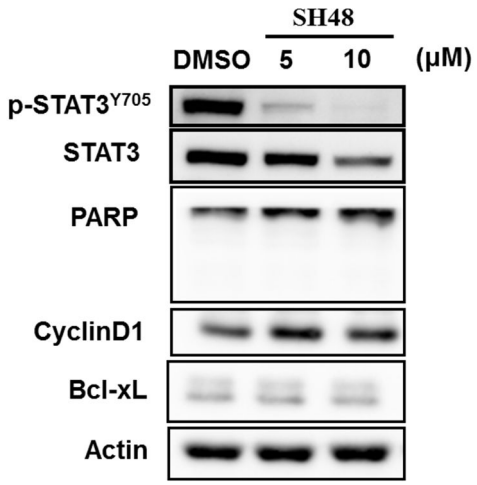


Fig. 3. SH48-induced cell death is unrelated to the apoptosis. MCF10A-*ras* cells were treated with SH48 for 24 h. Lysates were immunoblotted with the indicated antibodies.

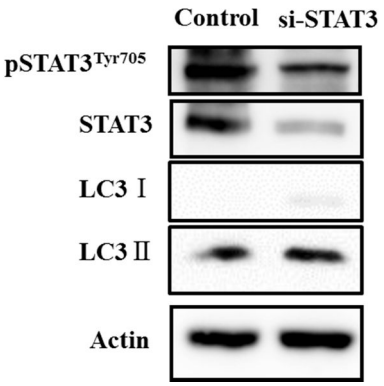


Fig. 4. STAT3 does not appear to regulate expression of LCI and LCII in breast cancer cells. MCF10A-*ras* cells were transfected with negative control or si-STAT3. The expression levels of proteins are determined by Western blot analysis.

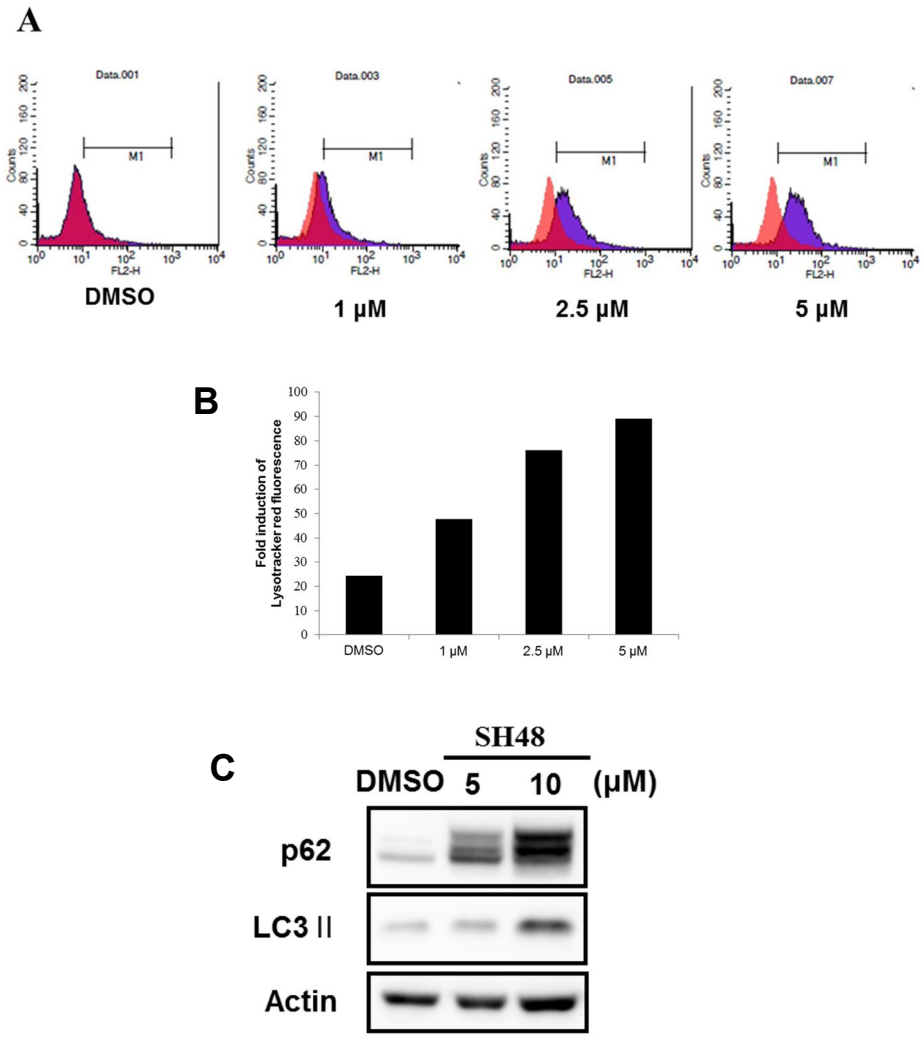


Fig. 5. Effect of SH48 on the induction of autophagic cell death in MCF10A-*ras* cells. MCF10A-*ras* cells were treated with indicated concentrations of SH48 for 24 h and then incubated with 100 nM LysoTracker Red for 30 min. The induction of cell death was measured by FACS analysis.

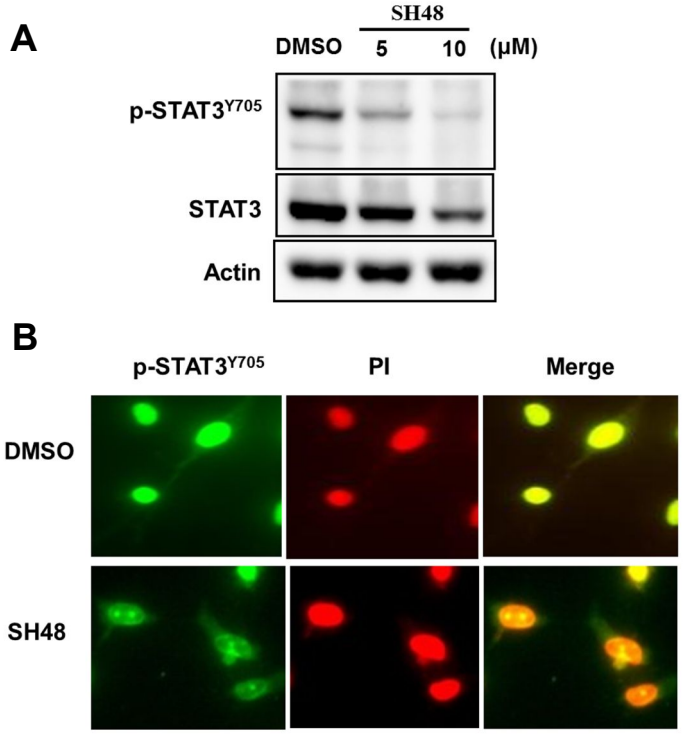


Fig. 6. SH48 suppresses STAT3^{Y705} phosphorylation and induces expression of the autophagy marker LC3Ⅱ. (A) MCF10A-*ras* cells were treated with SH48 for 24 h. Lysates were immunoblotted with the indicated antibodies. (B) Immunocytochemical analysis was performed using STAT3^{Y705} antibody after the treatment of MCF10A-*ras* cells with 10 μM SH48 for 24 h. Cells were stained with PI and visualized by confocal microscopy.

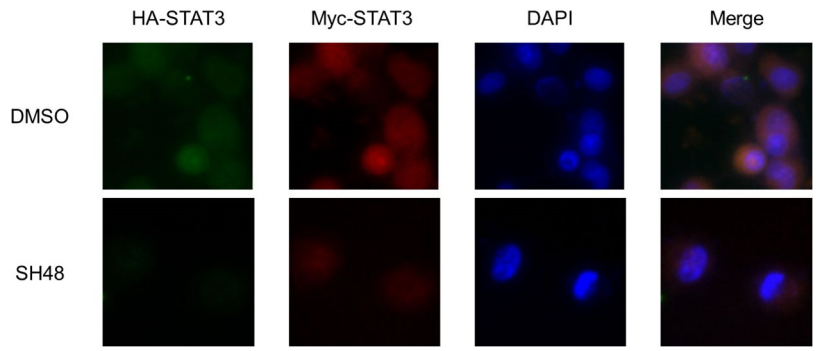


Fig. 7. SH48 inhibits STAT3 dimerization. Immunocytochemical analysis was performed using HA-tag and Myc-tag antibodies. MCF10A-*ras* cells were treated with 10 μM SH48 for 24 h. Cells were stained with DAPI and analyzed by confocal microscopy.

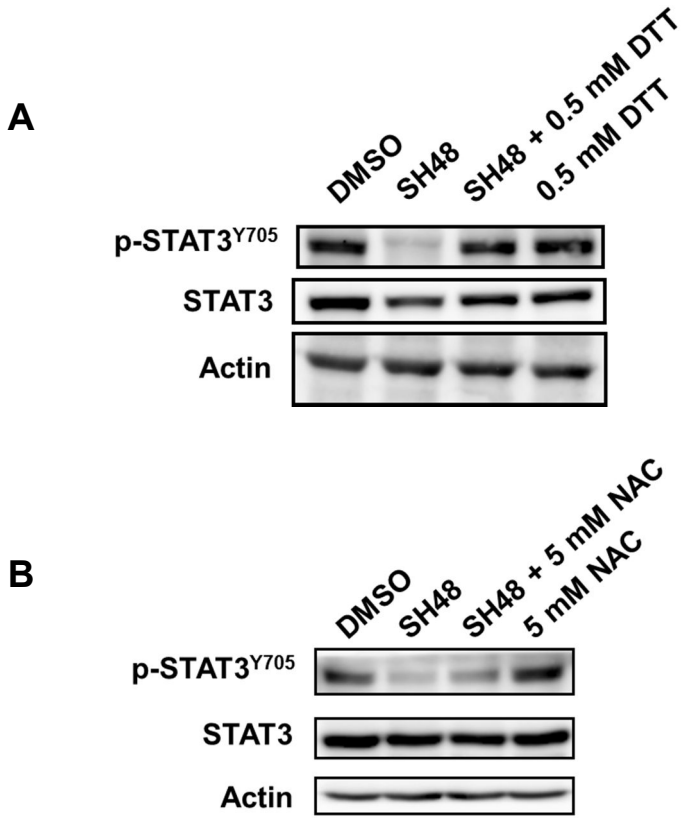


Fig. 8. Effects of the thiol reducing agents on SH48-induced STAT3 phosphorylation. MCF10A-*ras* cells pretreated with 0.5 mM DTT for 1 h, followed by 10 μM SH48 for 24 h. (B) MCF10A-*ras* cells treated with 5 mM NAC for 1 h, prior to exposure to 10 μM SH48 for 24 h.

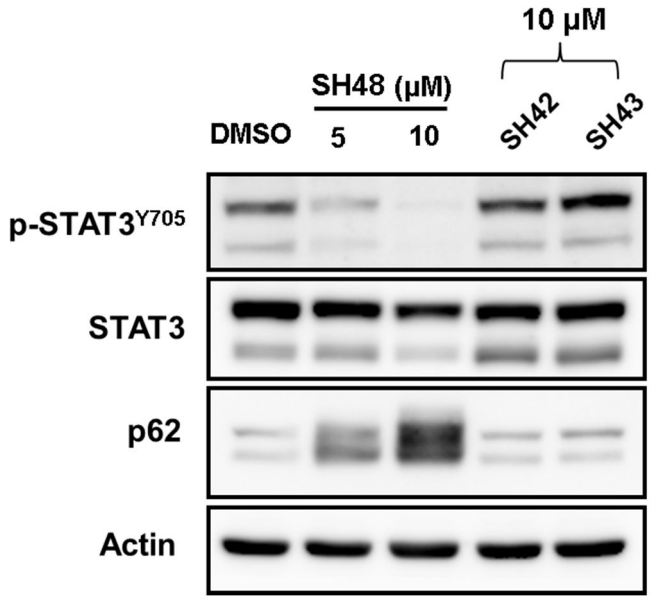


Fig. 9. SH48 inhibits STAT3 activation and upregulates p62. MCF10A-*ras* cells were treated with SH48 or 10 μM of SH42 and SH43 for 24 h.

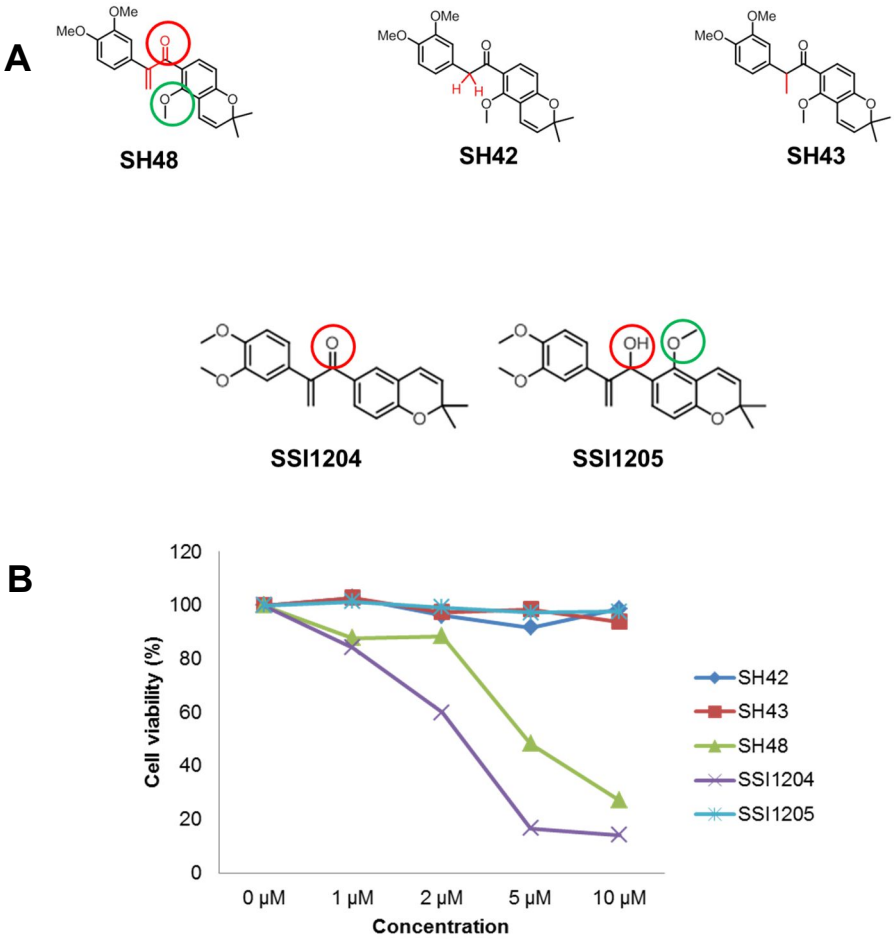
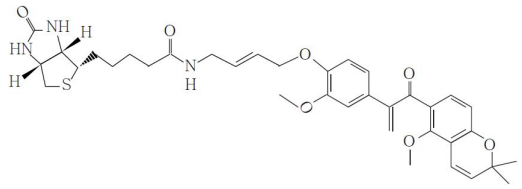
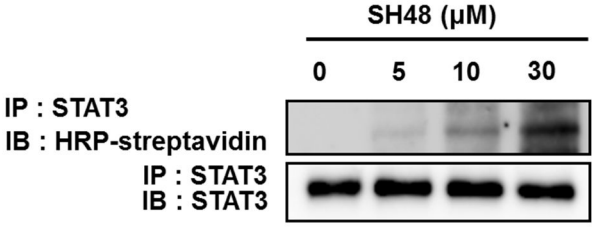


Fig. 10. The α,β-unsaturated carbonyl group is essential in suppressing the viability of MCF10A-*ras* cells by SH48 and SS1204. (A) The structures of SH42, SH43, SH48, SSI1204 and SSI1205 (B) MCF10A-*ras* cells treated with indicated concentration of SH42, SH43, SH48, SSI1204 and SSI1205 for 24 h.

A



B


SH48 (μM)
0
5
10
30
IP : STAT3
IB : HRP-streptavidin
IP : STAT3
IB : STAT3

C

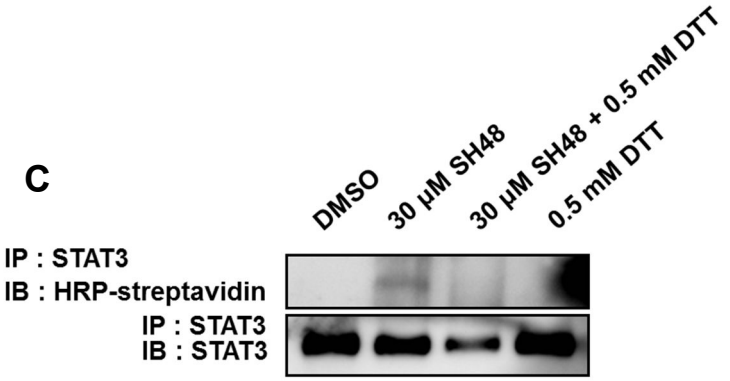

DMSO
30 μM SH48
30 μM SH48 + 0.5 mM DTT
0.5 mM DTT
IP : STAT3
IB : HRP-streptavidin
IP : STAT3
IB : STAT3

Fig. 11. SH48 binds directly to STAT3. (A) The Structure of biotinylated SH48. (B) MCF10A-*ras* cells were incubated with biotinylated SH48 for 24 h. The interaction between STAT3 and SH48 was assessed by immunoblot analysis, and the binding of biotinylated SH48 to STAT3 was detected with HRP-streptavidin. (C) MCF10A-*ras* cells were pretreated with 0.5 mM DTT for 1 h, and treated with biotinylated 30 μM SH48 for additional 24 h. The interaction between STAT3 and SH48 was assessed as described above.

Discussion

STAT3 is discovered in many cancer cells [21]. Overexpression of STAT3 participates in oncogenesis and tumor development. STAT3 is activated by phosphorylation and its dimerization. STAT3 dimer is translocated to nucleus and binds to a specific DNA site, thereby upregulating the transcription of target genes. Inhibition of abnormally elevated STAT3 activity or expression is an important therapeutic modality for malignancies, including breast cancer [22].

SH48 is a new synthetic analogue of deguelin that has a cancer chemopreventive and therapeutic effects [23]. SH48 has a structure similar to that of deguelin in the context of bearing an electrophilic α,β unsaturated carbonyl moiety . SH48 inhibits STAT3 signaling by direct-binding with STAT3, presumably at a critical thiol residue of STAT3. As SH48 binds to STAT3, it suppresses STAT3 signaling by inhibiting phosphorylation and subsequent dimerization. The dimerization of STAT3 is a crucial event for its activation. Therefore, the blockage of STAT3 dimerization suppresses oncogenesis.

It is well known that STAT3 is involved in transcriptional regulation of genes encoding apoptosis inhibitors (e.g., Bcl-x_L) and cell-cycle regulators

(e.g., cyclinD1) [24]. The inhibition of STAT3 induces apoptosis in breast cancer cells. However, SH48–induced suppression of STAT3 did not lead to apoptosis but rather induced autophagy in MCF10A-*ras* cells. The induction of autophagic cell death by SH48 was verified by upregulation of LC3Ⅱ and p62, which are the autophagy markers. LC3Ⅱ and p62 constitute autophagosome which is an important factor in autophagic signaling.

As autophagy is progressed, autophagosome is fused with lysosome and degraded. So, accumulation of p62 is is anticipated to be reduced as autophage is terminating. In line with this speculation, the expression level of p62 is increased till 24 hours and gradually decreased by 36 hours (S.-J. Hong, unpublished observation). When autophagosome is degraded, LC3Ⅱ maintains a constant level because LC3Ⅱ is degraded to a lesser extent than p62 during the destruction of autophagosome. A protease inhibitor (E64d) and a membrane permeable inhibitor (pepstatin A) attenuate the degradation of autophagosome, resulting in accumulation of autophagic markers [25].

There are several other autophagy markers such as beclin1, but we did not see any meaningful changes in the levels of this protein following SH48 treatment. Some autophagy occurs, independent of beclin1 accumulation [26]. We also measured the change of Atg5 and Atg7 mRNA levels, but the result is not clear. Again, SH48-induced autophagy may occur independent of these

markers [27]. Further studies will be necessary to further clarify this speculation.

SH48 has an α,β unsaturated carbonyl moiety which can mediate Michael addition between SH48 and STAT3.

Though our study suggests that SH48 inhibits STAT3 by Michael addition but hydrogen bonding may also be involved. It is a weak force but has enough energy for inducing reaction between molecules. Therefore, the cell viability was compared after treatment with the quimolar concentration of SH48 and SSI1205 incapable of interacting with STAT3 via H-bonding. However, H-bond is unlikely to contribute to inactivation of STAT3.

In summary, The synthetic deguelin analogue SH48 inhibits phosphorylation, DNA-binding activity, and transcriptional activity of STAT3, leading to the induction of autophagy in MCF10A-*ras* cells. SH48 induces autophagy in MCF10A-*ras* cells but not in MCF10A normal cells. Structure-activity analysis reveals that the α,β-unsaturated carbonyl moiety of SH48 is essential for its binding to STAT3 and consequent inactivation of this oncogenic transcription factor.

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국문초록

구조적으로 활성화된 signal transducer and activator of transcription 3 (STAT3)는 oncogenesis와 tumor development에 있어 중요한 역할을 한다고 보고되어 있다. 세포의 종류와 자극에 따라 STAT3는 apoptosis나 autophagy, 노화와 같은 anti-proliferative한 분화를 매개한다고 알려져 있다. STAT3는 tyrosine 잔기의 인산화에 의해 활성화되고, 이합체화반응을 일으키고, 핵 내로 이동한다. 본 연구에서는 새로운 STAT3 저해제인 SH48이 STAT3의 인산화를 억제하고, 핵 내 이동을 막아서 전사활동을 막는다는 사실을 발견하였다. SH48은 구조 내에 α,β-unsaturated carbonyl 그룹을 갖고 있고, 이 구조는 단백질의 thiol기와 반응한다. Thiol기를 환원시키는 물질인 dithiothreitol과 N-acetylcysteine은 SH48이 STAT3 활성을 억제하는 것을 억제하였다. 한편, SH48과 비슷한 구조를 갖지만 non-electrophilic한 화합물들은 STAT3 활성을 억제하지 못하였다. 바이오틴과 결합된 SH48로 실험을 진행한 결과, SH48이 STAT3에 직접 결합하는 것을 확인했다. SH48은 STAT3와 Michael addition을 통해 직접적으로 결합하고, STAT3 활성을 억제할 것으로 사료된다. SH48은 STAT3와 수소결합을 하지만, 이 상호작용은 STAT3의 인산화를 저해하지 않는다. SH48은 Ras가 transfection된 인체 유방상피세포인 MCF10A-*ras* 에서 세포 사멸을 일으키지만 일반 세포인 MCF10A 에서는 일으키지 않는다. SH48이 일으키는 세포사멸은 apoptosis가 아니고, LC3Ⅱ와 p62의 발

현을 증가시키는 것과 연관된 autophagy이다. SH48은 MCF10A-*ras*를 LysoTracker Red로 염색하여 FACS로 확인한 결과 autophagic 세포사멸 (typeⅡ 세포사멸)을 유도하는 것을 확인했다. 결과적으로, SH48은 STAT3를 직접적으로 목표로 하고 autophagy를 일으키는 것으로 사료된다.