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The mechanistic study of a novel compound on the inhibition of neoplastic cell transformation and vemurafenib resistance

조선대학교 대학원

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이 논문을 약학 석사학위신청 논문으로 제출함

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
BME	Basal medium Eagle
BrdU	5-bromo-2'-deoxyuridine
c-Fos	AP-1 trascription factor subunit C
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinase1/2
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MEK1/2	Mitogen-activated protein kinase-extracellular signal-regulated kinase
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
RAF	Rapidly accelerated fibrosarcoma
RTK	Receptor tyrosine kinase
RIPA buffer	Radioimmunoprecipitation assay buffer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOFT AGAR	Anchorage-independent cell transformation

국문 초록

신생물 세포형질전환 및 vemurafenib 내성을 억제하는 신규 화합물의 기전 연구

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지도교수: 최홍석

조선대학교 대학원 약학과

악성 흑색종(malignant melanoma)은 멜라닌 세포의 악성화에 의해 유발되는 종양으로 주로 피부에서 발생한다. 가장 악성도가 높고 위험한 형태의 암으로 진행속도가 매우 빠르며 최근 사망률이 증가 하는 추세를 보이고 있다. 이를 치료하기 위해 악성흑색종 환자의 대부분이 가지고 있는 BRAF의 돌연변이, BRAF V600E를 표적으로 하는 가장 대표적이고 특이적인 약물인 vemurafenib가 개발되었고 널리 사용 되고 있다. 하지만 vemurafenib를 지속적으로 투여했을 경우 MAPK의 재활성이 발생하게 되어 약물에 대한 저항성이 쉽게 발생하는 것이 문제로 대두 되고 있다. 따라서 획득 된 약물에 대한 저항성은 중요한 문제가 되었으며, 이를 극복하기 위한 다양한 조절 기전에 대한 이해와 연구 그리고 새로운 치료 전략 및 효과적인 신규화합물이 필요한 실정이다.

이러한 배경으로 본 연구에서는 vemurafenib의 유도체로써 개발된 신규 화합물인 KS28이 상피세포에서 EGF에 의해 유도된 세포형질전환 억제와, vemurafenib 저항

성이 발생한 흑색종 세포에서 미치는 영향 및 분자적 메커니즘을 규명하고자 하였다. 먼저 상피세포에서 KS28에 대한 연구를 진행하였는데, KS28은 JB6 Cl41에서 세포 생존과 성장을 억제함은 물론 epidermal growth factor (EGF)에 의해 유도된 MAPK, c-Fos, JNK, c-Jun의 단백질 인산화를 억제 하였다. 또한 EGF에 의해 유도된 c-Fos와 c-Jun 그리고 AP-1 promoter 활성까지도 농도 의존적으로 억제 하는 것을 볼 수 있었으며 EGF에 의해 유도되는 상피세포 형질전환도 억제 하였다. 이러한 결과는 KS28가 MAPK 및 JNK 신호경로를 차단시킴으로써 신생물 세포형질전환을 억제 할 수 있음을 나타냈다.

이후 vemurafenib 저항성을 갖는 흑색종 세포에서의 효과를 확인하기 위해서, 흑색종 세포에 vemurafenib를 지속적으로 처리하여 저항성 세포를 구축하였고 이 저항성 세포에서 MAPK의 재활성, 그리고 c-Fos, JNK 및 c-Jun의 발현이 증가되는 것을 확인하였다. 이렇게 획득된 저항성을 극복하는 방법으로써 최근 연구에서는 vemurafenib와 MEK 억제제와 같은 다른 분자 표적제와의 병용요법이 저항성 세포에서 치료 효능을 증가시킬 수 있다고 보고 되었다. 본 연구에서는 KS28의 단독요법이 A375R 세포에서 vemurafenib와 MEK 억제제인 PD98059의 병용요법보다 뛰어난 세포 생존력 억제와 ERK의 인산화 발현을 억제에 더 효과적임 보였다. 따라서 새로운 화합물이 vemurafenib 저항성을 극복하기 위한 치료제로써의 잠재력을 확인할 수 있었다. 이후 KS28의 분자적 메커니즘을 조사 한 결과 KS28은 MAPK의 재활성 및 c-Fos 단백질 인산화를 억제하였으며 c-Fos 와 AP-1 promoter 활성을 농도 의존적으로 억제 시켰다. 또한 soft agar matrix에서도 종양 생장을 매우 효과적으로 억제하는 것을 관찰 할 수 있었다.

종합적으로, 위와 같은 결과를 통하여 KS28 화합물이 다양한 세포기능을 하는 MAPK의 신호경로를 차단시킴으로써 vemurafenib 저항성을 극복 할 수 있고 신규 화합물로써의 가능성을 제안한다.

I. Introduction

Receptor tyrosine kinases (RTKs), such as a promising class of receptors, have been extensively researched in the last three decades. These are one of the essential mediators of cell signaling mechanism for various cellular processes such as tumor growth, progression and metastasis [1]. Overexpression or structural alterations of RTKs are often associated with human cancers and tumor cells are known to use RTK transduction pathways to achieve cellular processes [2]. In addition, overexpression of RTKs, such as epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR), can induce RAS activation and further contribute to cell transformation and tumorigenesis. Among the various pathways that are activated by RAS, the pathway that contains activated protein kinases RAF, MEK, and ERK has been found to promote cell survival and inhibit apoptosis [3]. In this way, the majority of human tumors depend on activation of RAS and RAF signaling pathways to achieve cellular proliferation and survival [4].

Of these signaling pathways, the RAF protein kinases are key intermediates in cellular signal transduction and these are best known for their role that widely utilized to control many cellular processes, including proliferation, differentiation, and survival [5]. The three types of RAF proteins (A, B and CRAF) can be activated by RAS and exert both kinase-dependent and kinase-independent, tumor-promoting functions. The kinase-dependent functions are primarily mediated by the MEK / ERK pathway and activation of this pathway is associated with proliferation in human tumors [6]. C-Raf (also known as Raf-1) was identified both as an interaction partner and activator of mitogen-activated protein kinase kinase (MEK), the dual-specificity kinase responsible for activation of extracellular signal-regulated kinase (ERK). B-Raf is the family member most easily activated by Ras, and the basal kinase activity of B-Raf is higher than that of C-Raf and A-Raf [7]. This provides a potential rationale for frequent mutational activation of BRAF other than CRAF or ARAF observed in human tumors [8].

Approximately 66 % of melanoma patients were reported to have mutations in BRAF (BRAF V600E). Melanoma is one of the most dangerous cancers and responsible for the majority of skin cancer deaths [9]. The incidence of melanoma has risen rapidly in the last three decades and has become a significant health risk worldwide [10]. The most common activating mutation in BRAF is the substitution of glutamic acid for valine at position 600 (V600E mutation), resulting in constitutive activation of the mitogen-activated protein kinase (MAPK) pathway. Thus, several BRAF inhibitors have been developed and undergoing preclinical and clinical research. These findings led to the FDA (Food and Drug Administration) approval of vemurafenib in 2011 and a dramatic improvement in the standard of care for these patients [11]. Although vemurafenib have shown effectiveness in melanoma cell lines which have mutations of BRAF, acquired drug resistance frequently develops after 6 to 8 months of starting therapy due to development of resistance mechanisms [12]. There are various mechanisms of resistance that include activation of alternative signaling pathways as well as reactivation of the MAPK pathway. The precise and detailed molecular mechanisms, which explain vemurafenib resistant in melanoma through these alternative survival pathways, are poorly described. However, previous studies have shown that reactivation of the MAPK pathway has taken significance in vemurafenib resistance [13].

As a way of overcoming resistance, recent research has shown that an approach to simultaneously inhibiting BRAF V600E and MEK is highly effective in overcoming resistance [14]. However, resistance also was developed soon against the combination of BRAF V600E and MEK inhibitors as well. Therefore, understanding and research on various control mechanisms and effective new compounds are needed to overcome vemurafenib resistance. In this study, we revealed that KS28, a novel derivative of vemurafenib, can inhibit MAPK pathway in resistant cells to recover from vemurafenib resistance. Given that this novel compound is effective as single treatment, our study suggests a novel chemotherapeutic strategy to overcome vemurafenib resistance by avoiding the toxicities associated with combinatorial chemotherapy. Furthermore, it can provide new insights and applicability in the treatment and prevention of tumors induced by EGF.

II. Materials & Methods

1. Materials

1.1 Reagents and antibodies

Dulbecoo`s modificed Eagle`s medium (DMEM), Eagle`s minimal essential medium (MEM), fetal bovine serum (FBS), L-glutamine, gentamicin and FBS were purchased from Invitrogen (Carlsbad, CA, USA). 3-[4,5-Dimethlthiazol-2-thiazoyl]-2,5-diphenyltetrazolium bromide (MTT) and β-actin were from Sigma-Aldrich. Cell proliferation ELISA, BrdU (colorimetric) kit was from Roche Applied Science (Indianapolis, IN, U.S.A.). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Billerica, MA, USA). Antibodies against phospho-MEK1/2, -ERK1/2, -JNK1/2, -c-Fos, -c-Jun, Total-MEK1/2, ERK1/2, JNK1/2, c-Fos and c-Jun were purchased from Cell Signaling Technology (Beverly, MA). The jetPEI cationic polymer transfection reagent was from Polyplus-Transfection (New York, NY, USA). The Dual-luciferase reporter assay kit was purchased from Promega (Madison, WI, U.S.A.).

1.2 Cell culture and establishment of resistant cell lines

A375 cells and JB6 Cl41 mouse epidermal cells were purchased from American Type Culture Collection (ATCC). To generate cell lines with *in vitro* resistance, the melanoma cells were treated with 5 μM vemurafenib for 1 hour, and then surviving cells were cultured with 5 μM vemurafenib until a vemurafenib-resistant sub-line was established. JB6 Cl41 cells were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum and A375 & A375R cell cultures were cultured in DMEM supplemented with 10% fetal bovine serum. All cell lines were cultured and maintained at 37°C in humidified air containing 5% CO_2.

2. Methods

2.1 MTT assay

3-[4,5-Dimethylthiazol-2-thiazolyl]-2,5-diphenyltetrazolium Bromide (MTT) Assay was performed to estimate cell viability. Cells were seeded (5×10^4 cells/mL) in 96-well plates with 100 μl of cell suspension in each well. After culturing for 24 h, cells were treated with different concentrations of KS-28. The cells were then treated with 5 μg/mL MTT solution (10 μL/well) and incubated for 4 h, and absorbance was measured at 450nm using a VersaMax ELISA microplate reader.

2.2 Cell proliferation assay

Cells were seeded (3×10^5 cells/mL) in 96-well plates in 100 μl of 5% FBS-MEM. After 24h, the cells were treated or not treated with 10 ng/mL EGF with KS-28 for 48 h, labeled with 10 μL/ well BrdU labeling solution, and then reincubated for additional 4 h at 37°C in a 5% CO_2 atmosphere. After the media was removed, FixDenat solution was added to each well and incubated at room temperature (RT) for 30 min. After 30 min, FixDenat solution was removed, and anti-BrdU-POD working solution was added to each well and incubated for 90 min at RT. The cells were then washed with washing solution for 3 times and 100 μL of substrate solution was added in each well and incubated for 30 min. Cell proliferations was estimated by measuring the absorbance at 370 nm.

2.3 Protein immunoblot analysis

For immunoblotting, The Cells were harvested and disrupted in RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH7.4), 0.25% Sodium deoxycholate, 1 mM EDTA, 1% NP40, 1 mM NaF, 0.2 mM PMSF, 0.1 mM Sodium orthovanadate and protease inhibitor cocktail (Roche). Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membranes. The membranes were blocked and probed with indicated primary antibody, overnight at 4 ˚C. After hybridization with HRP (horseradish

peroxidase)-conjugated secondary antibody from rabbits or mice, The protein bands were visualized using a SuperSignal West Femto chemiluminescence substrate (Pierce) and The Amersham Imager 680 was used for chemiluminescence detection.

2.4 Reporter gene assay

For detecting firefly luciferase activity, the reporter gene assay was performed using lysates from AP-1-, c-jun-, c-fos-, or AP-1-luc-transfected JB6 Cl41, A375 and A375R cells. The reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line and the renilla luciferase activity generated by this vector was used to normalize the results for transfection efficiency. Cell lysates were prepared by first washing the transfected JB6 Cl41 cells once with phosphate-buffered saline (PBS) at RT. After removing the PBS completely, passive lysis buffer (PLB, Promega) was added, and then cells were incubated at RT for 1 h with gentle shaking. The supernatant fraction was used to measure firefly and renilla luciferase activities. Cell lysates were mixed with luciferase assay II reagent, and firefly luciferase light emission was measured by GloMax®-multi detection system (Promega). Subsequently, renilla luciferase substrate was added in order to normalize the firefly luciferase data. The c-fos-luc promoter (pFos-WT GL3) and c-jun-luc promoter (JC6GL3) constructs were kindly provided by Dr Ron Prywes (Columbia University, New York, NY). The AP-1 luciferase reporter plasmid (−73/+63 collagenase-luciferase) was kindly provided by Dr Dong Zigang (Hormel Institute, University of Minnesota, Austin, MN).

2.5 Anchorage-independent growth assay (Soft agar assay)

The effect of KS28 was investigated in A375, A375R cells and also the effect of KS28 in EGF induced cell transformation was also investigated in JB6 Cl41 cells. Cells were exposed to the indicated drug in 1 ml of 0.3% basal medium Eagle`s (BME) containing 10% FBS, 2 mM L-glutamine and 25 μg/mL gentamicin. The cultures were maintained at 37℃ in 5% CO_2 incubator for 10-17 days. The cell colonies were scored using and Axiovert 200M fluorescence microscope and Axio Vision software (Carl Zeiss, Thornwood, NY

III. Results

1. KS28 inhibits cell viability and EGF-induced cell proliferation of JB6 Cl41 cells

Vemurafenib (PLX4032) is a synthetic molecule, which was potent inhibitor of oncogenic B-RAF kinase activity in melanoma and represents a new standard of care in patients with advanced melanoma harboring a BRAF-V600E mutation. A new synthetic compound, KS28 was designed and synthesized as a derivative of vemurafenib, which inhibits MAPK signaling. And then we evaluated the effects of KS28 on the cell viability and cell proliferation of JB6 Cl41 mouse epidermal cells by MTT assay and BrdU incorporation assay, respectively. KS28 at lower concentrations from 0.01 μM to 0.1 μM did not affect cell viability, whereas considerably decreased the cell viability of JB6 Cl41 cells at higher concentrations more than 0.5 μM (Figure 1B). However, the inhibitory effect of KS28 at high concentrations (>3 μM) may be a result of cytotoxicity. Therefore, we examined the inhibitory effect of KS28 on cell proliferation induced by epidermal growth factor (EGF) that can induce cell transformation in JB6 Cl41 cells. JB6 Cl41 cells were treated with EGF in absence or presence of KS28. As shown in Figure 1C, KS28 was significantly effective in suppressing the EGF-induced cell proliferation, at concentrations from 0.5 uM to 1uM.

Figure 1

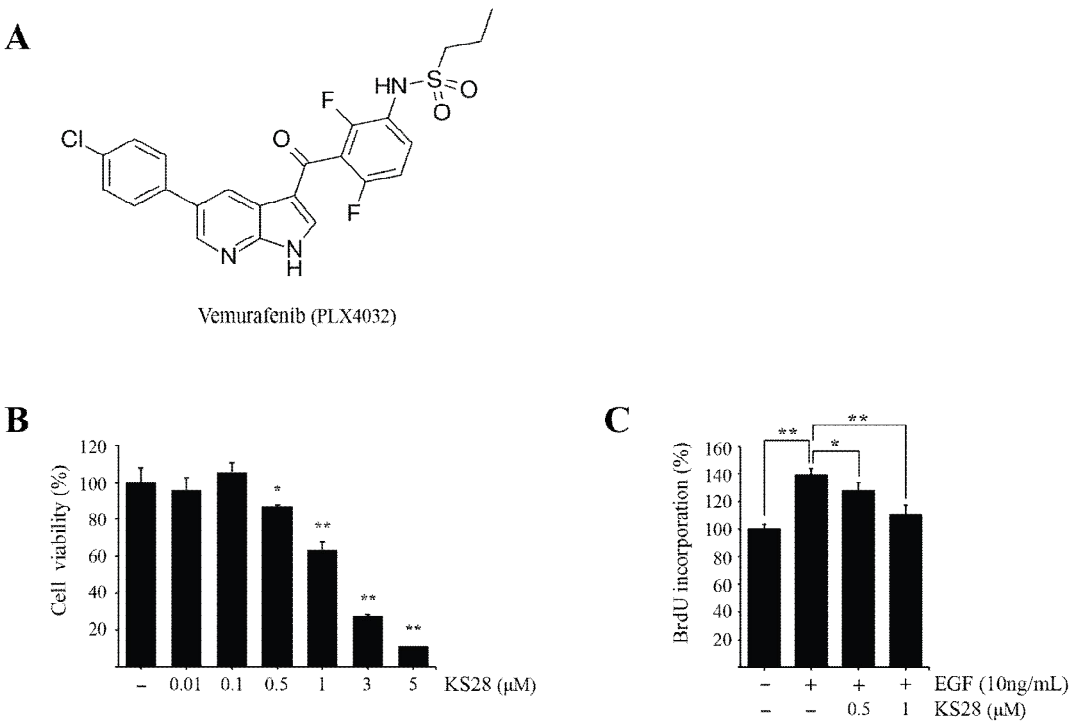


Figure 1. Inhibitory effects of KS28 on cell viability and cell proliferation of JB6 Cl41 cells

(A) Chemical structure of Vemurafenib. (B) JB6 Cl41 cells were seeded in 96-wells and cultured for 48h at 37°C in 5% CO_2 atmosphere and then cells were treated with KS28 dose-dependently as indicated concentration. Cell viability was measured using a MTT assay. The results represent means ± S.D. standard deviations of triplicate experiments compared with control cells (*p<0.05; **P<0.01). (C) Cells were treated with the 10ng/ml EGF or KS28, alone or combinations, for 72h, and then cell proliferation was estimated by BrdU incorporation assay (*p<0.05; **P<0.01).

2. KS28 suppresses signaling cascade of MEK/ERK, c-Fos and JNK/c-Jun induced by EGF in JB6 Cl41 cells

The mitogen-activated protein kinases (MAPKs) signaling is one of the most important protein kinase cascades activated by epidermal growth factor (EGF) [1]. We hypothesized that KS28 might inhibit this signaling pathway. Therefore, in this study, we investigated the inhibitory effects of KS28 on the EGF-induced phosphorylation of MAPK signaling in mouse skin epidermal JB6 Cl41 cells. JB6 Cl41 cells were treated with 10ng/ml EGF for 15 min, in the absence or presence of various concentrations of KS28 and immunoblotted with the anti-phospho antibodies against MEK1/2, ERK1/2, p90RSK, and c-Fos, respectively. Results indicated that treatment of KS28 significantly decreased the EGF-induced phosphorylation of MEK1/2, ERK1/2, p90RSK, c-Fos (Figure 2A, 2B). Constitutively active ERK signaling pathway upregulates JNK and activates c-Jun oncogene [15]. Therefore, we examined the inhibitory effects of KS28 on the JNK pathways induced by EGF, such as JNK1/2 and c-Jun of JB6 Cl41 cells. The results showed that treatment of KS28 decreased the EGF-induced phosphorylation of JNK1/2 as well as c-Jun that may lead to downregulation of c-Jun transcriptional activity (Figure 2C). Taken together, these data suggest that KS28 has inhibitory effect on the signaling pathway of MAPK and JNK signaling.

Figure 2

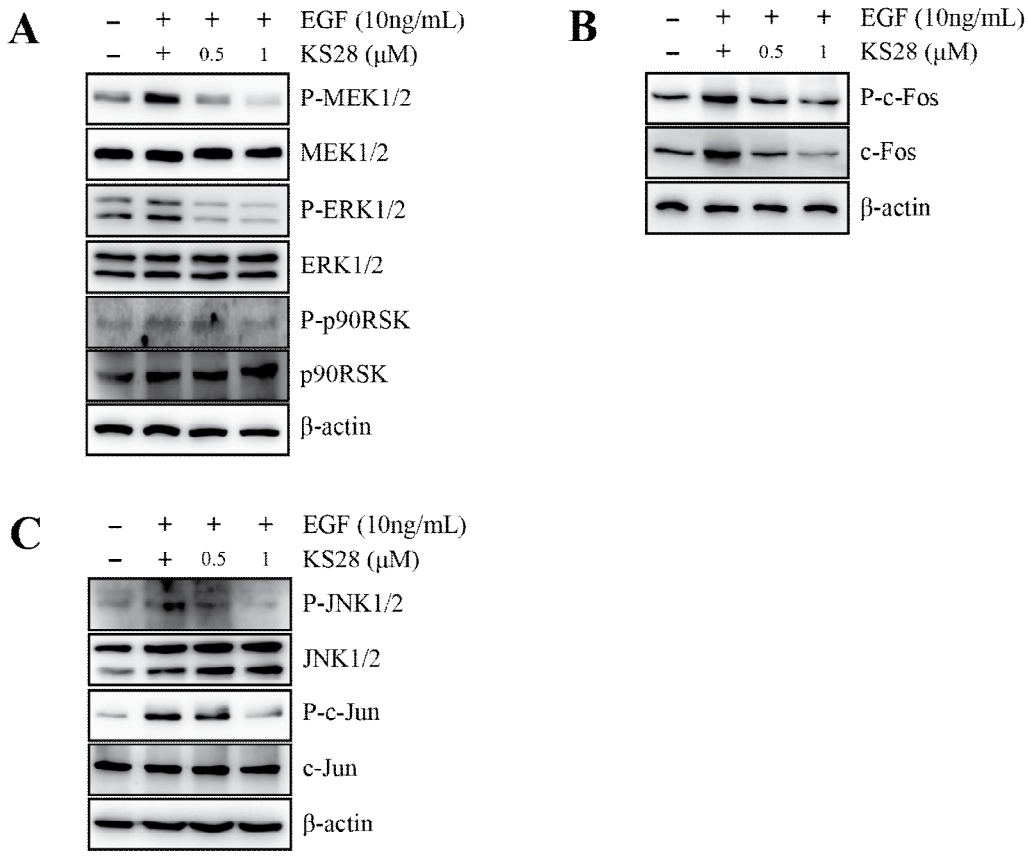


Figure 2. Inhibitory effects of KS28 on EGF-induced phosphorylation of MAPKs, c-Fos, JNK and c-Jun in JB6 Cl41 cells

(A-C) Cells were serum starved for 24h and treated with the indicated doses of KS28 for 24h, then exposed to 10ng/ml EGF for 15min. Cells were harvested and proteins in whole cell lysates were separated by SDS-PAGE and then immunoblotting analysis was performed using specific antibodies against each of proteins. The levels of phosphorylated and total proteins related with MEK-ERK (A), c-Fos (B), and JNK-c-Jun (C) signaling cascades. Values represent means ± S.D. standard deviations of triplicate experiments compared with control cells and EGF-treated cells (*$p<0.05$; **$P<0.01$).

3. KS28 inhibits EGF-Induced *c-fos*, *c-jun* and AP-1 promoter activity in JB6 Cl41 cells

c-Fos is proto-oncogene and forms heterodimer in combination with c-jun that is oncogenic transcription factors activated by MAPK signaling pathway. As a result, complex forms AP-1 (Activator Protein-1), which binds DNA at the promoter and enhancer regions of target genes and converts extracellular signals into changes of gene expression [16]. Therefore, to investigate whether effect of KS28 regulated epidermal growth factor (EGF)-induced c-fos, c-jun and AP-1 transcriptional activity, we examined the effect of EGF on the c-fos, c-jun and AP-1 promoter. The results showed that treatment with EGF was found to significantly induce AP-1 transactivation in JB6 Cl41 cells. And KS28 treatment inhibited the EGF-induced c-fos, c-jun and AP-1 promoter activity (Figure 3). Taken together, these results support the idea that suppression of the c-fos, c-jun and AP-1 promoter activity induced by EGF is one of the mechanisms on the anti-proliferative effect of KS28

Figure 3

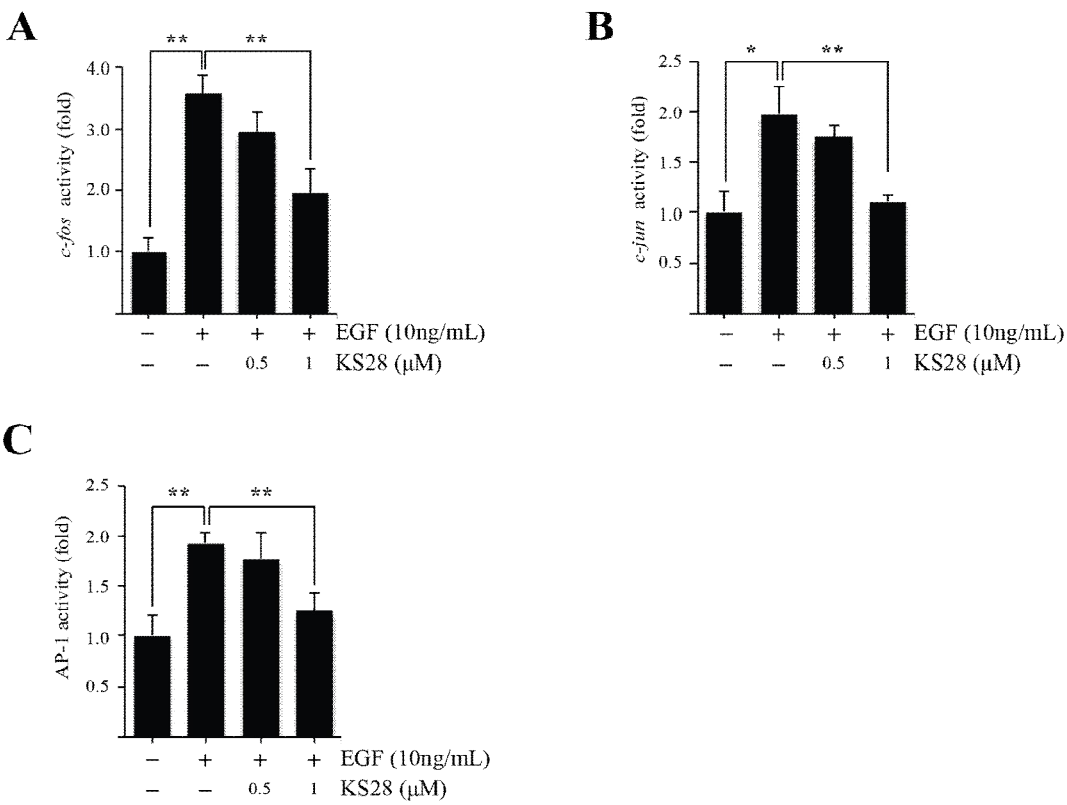


Figure 3. Inhibitory effects of KS28 on EGF-induced c-fos, c-jun and AP-1 promoter activity in JB6 Cl41 cells

(A-C) Cells were seeded and transfected with the luciferase reporters, *c-fos-luc* (A), *c-jun-luc* (B), and AP-1-luc (C) promoter gene, respectively, with the pRL-TK vector. After transfection for 24h, cells were serum starved for 12h, and then treated with indicated doses of KS28 for 24h with exposed or not exposed with 10ng/ml EGF. In all of the promoter assays, the firefly luciferase activity was determined in cell lysates and normalized against renilla luciferase activity, and these luciferase activities are expressed relative to control cells. Values represent means ± S.D. standard deviations of triplicate experiments compared with control cells and EGF-treated cells (*$p<0.05$; **$P<0.01$).

4. KS28 suppresses anchorage-independent cell transformation induced by EGF in JB6 Cl41 cells

AP-1 is a dimeric complex of homodimers or heterodimers of Jun, Fos and major transcription factor involved in neoplastic cell transformation of JB6 Cl41 cells induced by various tumor promoters [17]. We examined that KS28 can prevent cell transformation in JB6 Cl41 cells induced by EGF. JB6 Cl41 cells were treated separately with 10 ng/mL EGF in the absence or presence of various concentrations of KS28 in a soft agar matrix and incubated at 37°C in a 5% CO_2 incubator for 13 days. The results showed that KS28 significantly inhibited the EGF-induced cell transformation of JB6 Cl41 cells in a dose-dependent manner (Figure 4). Overall, these data suggested that KS28 can suppress tumor development of neoplastic cell transformation in epidermal mouse skin cells stimulated with EGF.

Figure 4

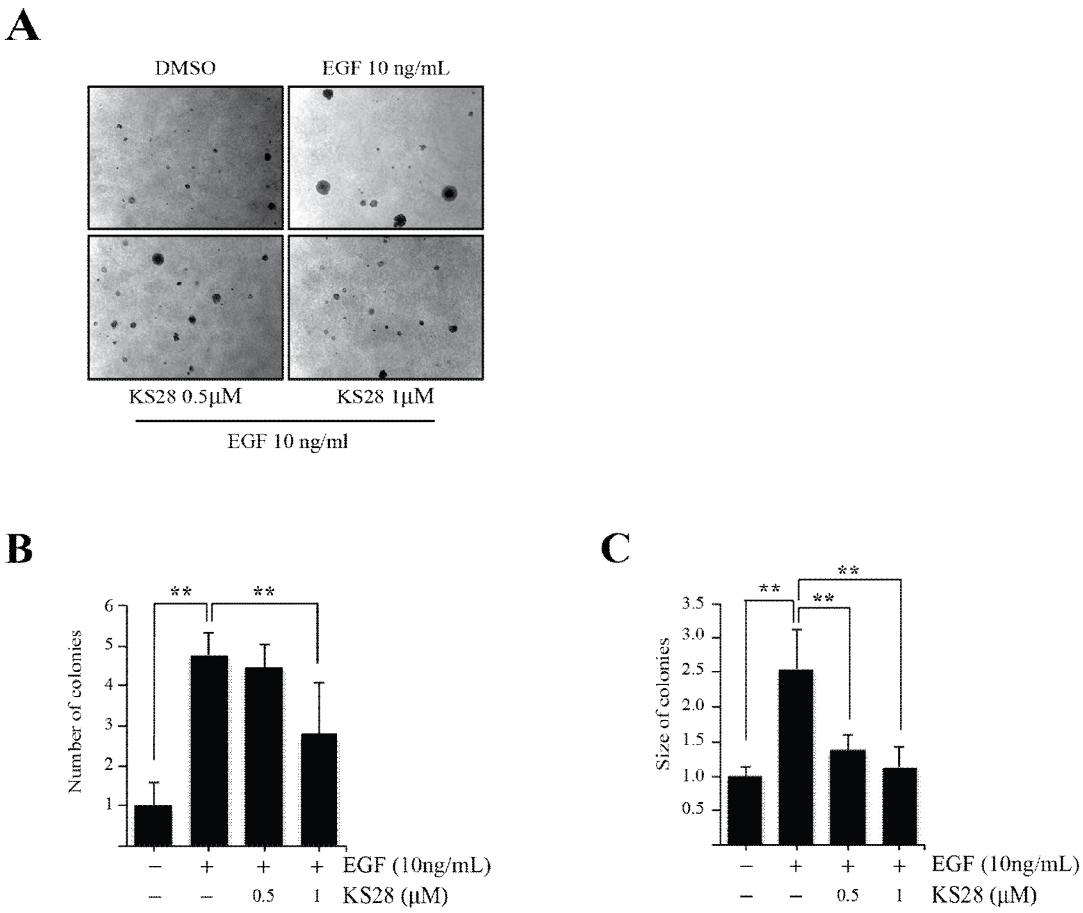


Figure 4. Inhibitory effects of KS28 on EGF-induced neoplastic cell transformation in JB6 Cl41 cells

The cells were treated with 10 ng/ml of EGF in absence or presence of KS28 in soft agar, incubated at 37°C in 5% CO_2 atmosphere for 13 days. The representative colonies from three separate experiments were photographed (A) and average number of colonies and size were measured under a microscope with the aid of the Image-Pro Plus software program (B, C). Values represent means ± S.D. standard deviations of triplicate experiments compared with control cells and EGF-treated cells (*$p<0.05$; **$P<0.01$).

5. Vemurafenib resistant melanoma cell display enhanced MAPK, c-Fos, and c-Jun signaling

In the present study, to analyze the mechanism of resistance to vemurafenib, we established a cellular model of acquired resistance to vemurafenib in melanoma cell line A375. To establish A375R, parental A375 cells were continuously exposed by gradually increasing concentrations of vemurafenib (up to 5 μM concentration) over a period of 4 months. To confirm vemurafenib resistance, we evaluated the inhibitory effect of vemurafenib on the cell viability of A375 and A375R cells by MTT assay. As expected, results showed that A375R cells were resistant to vemurafenib (IC_{50} > 10 μM) compared with A375 (IC_{50} = 0.8 μM). Next, we compared the expression level of MAPK signaling in these cells by immunoblotting. As a result, we found that basal level of phosphorylated CRAF, MEK, ERK, p90RSK, c-Fos, JNK and c-Jun signaling in A375R is elevated. Taken together, these data confirm that MAPK signaling is reactivated in vemurafenib resistant melanoma cells.

Figure 5

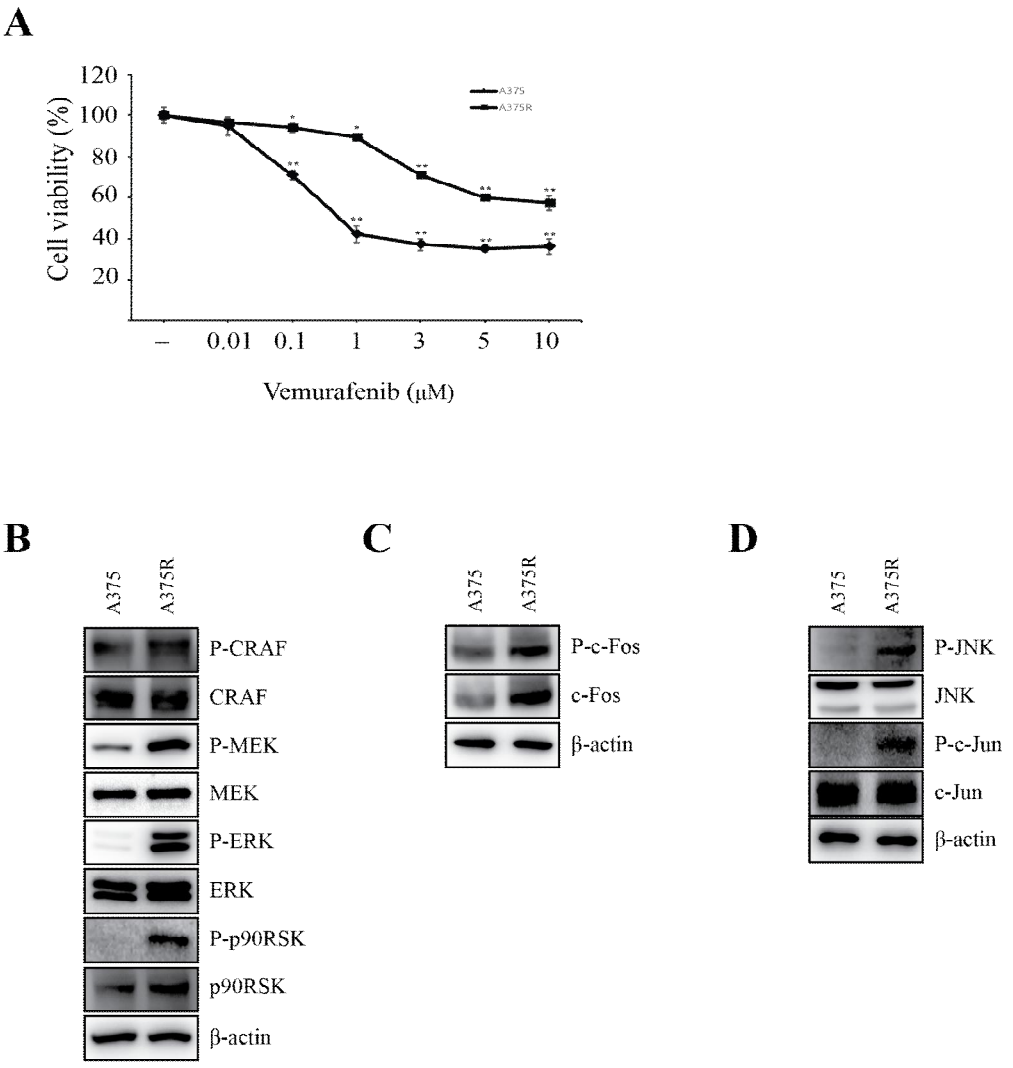


Figure 5. Establishment of vemurafenib resistant melanoma cell and reactivation of MAPK signaling pathway

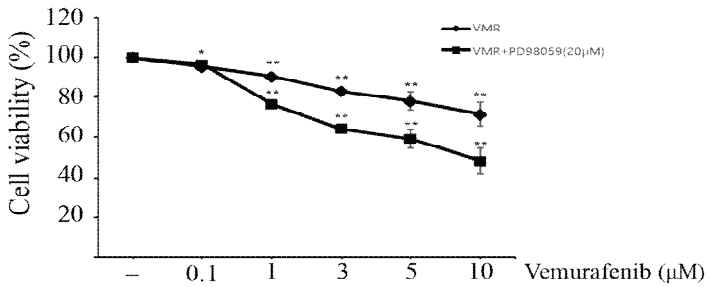
(A) A375 and A375R cells were seeded in 96-wells and cultured for 48h at 37°C in 5% CO_2 atmosphere and then cells were treated with various concentrations of vemurafenib as indicated concentration. Cell viability was estimated using a MTT assay. Values represent means ± S.D. standard deviations of triplicate experiments compared with control cells (*$p<0.05$; **$P<0.01$). (B-D) A375 and A375R cells were seeded and cultured for 48h and then cell were harvested, lysed and immunoblotted using respective antibodies.

6. KS28 inhibits cell viability and MAPK signaling compare with combined treatment of vemurafenib and PD98059 in vemurafenib resistant cell

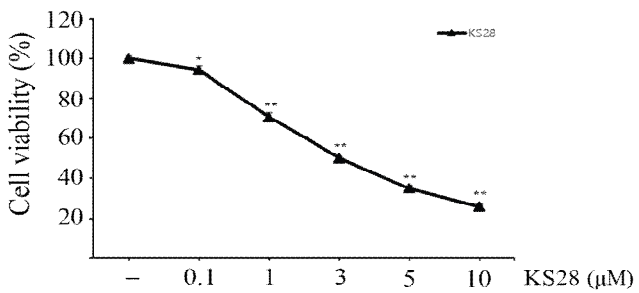
Combination therapy with vemurafenib and other molecular targeting agents, such as MEK inhibitors, is known to prolong the overall survival of treated patients and increase the efficacy of treatment in cancer [18]. To compare the inhibitory effects between KS28 and combination therapy, we analyzed cell viability treated by either KS28 alone or by the combination of vemurafenib and PD98059, a MEK1/2 inhibitor. As shown in Figure 6A, the treatment with combination of vemurafenib and PD98059 inhibited cell viability of A375R cells. The treatment with KS28 alone has more inhibitory effect on the cell viability of A375R cells than combination treatment (Figure 6B). Next, to investigate the inhibitory effects of KS28 alone and in combination with vemurafenib and PD98059, we analyzed changes in the MAPK signaling pathway using western blotting. As a result, KS28 more inhibited phosphorylation of ERK than combination treatment. These are implying that treatment of KS28 alone may overcome resistance to vemurafenib in human melanoma cells.

Figure 6

A



B



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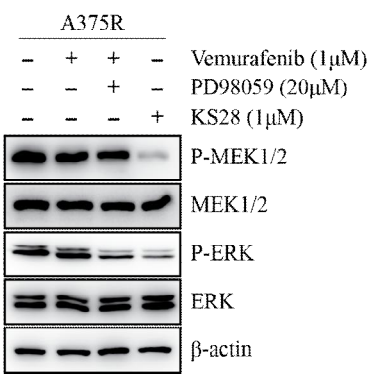

Figure 6. Inhibitory effects of KS28 and combined treatment with vemurafenib and PD98059 in A375R

A375R cells were seeded in 96-wells and cultured for 48h at 37°C in 5% CO2 atmosphere. Cells were treated with vemurafenib alone or in combination with PD98059 (20μM) (A) and KS28 dose-dependently as indicated concentration (B). Cell viability was measured using a MTT assay. The results represent means ± S.D. standard deviations of triplicate experiments compared with control cells (*$p<0.05$; **$P<0.01$). (C) A375R cell were seeded and cultured for 24h at 37°C in 5% CO_2 atmosphere. Then cells were treated with vemurafenib and vemruafenib with PD98059 and KS28 for 24h. Cells were harvested and proteins in whole-cell lysates were separated by SDS-PAGE and immunoblotted using respective antibodies.

7. KS28 inhibits phosphorylation of MAPKs signaling and c-Fos in vemurafenib resistant cells

Previous studies have shown that reactivation of the MAPK pathway has taken significance in vemurafenib resistance. Therefore, to confirm whether it is possible to overcome resistance by KS28, we examined the level of phosphorylated MEK1/2, ERK1/2, p90RSK and c-Fos after treating with vemurafenib and KS28. The results showed that treatment with vemurafenib and KS28 decreased the levels of phosphorylated MEK1/2, ERK1/2, p90RSK, and c-Fos in A375 cells. Treatment with vemurafenib did not affect the levels of phosphorylated MEK1/2, ERK1/2, p90RSK, and c-Fos in vemurafenib resistant cells, whereas treatment with KS28 induced significant decrease (Figure 7A, 7B). Accordingly, these data suggested that KS28 has inhibitory effect in vemurafenib resistant cells through the downregulation of MAPK signaling pathway.

Figure 7

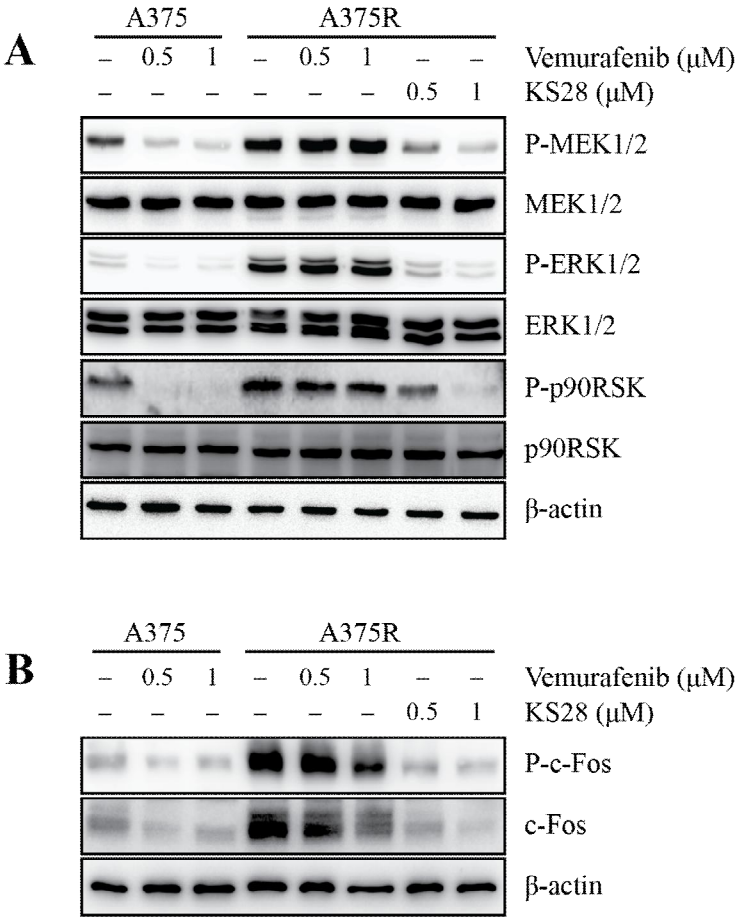

Figure 7. Inhibitory effects of KS28 on phosphorylation of MAPKs and c-Fos in A375R cells

(A-B) A375 and A375R cells were seeded and cultured for 24h at 37°C in 5% CO_2 atmosphere. Then cells were treated with vemurafenib and KS28 for 24h. Cells were harvested and proteins in whole-cell lysates were separated by SDS-PAGE and immunoblotted using respective antibodies.

8. KS28 inhibits *c-fos* and AP-1 transcriptional activity in vemurafenib resistant cells

c-Fos is in part of AP-1 proteins which can transform cells efficiently in culture and induce target-gene transcription. And the increased AP-1 is induced by several external stimuli that increase mitogen-activated protein kinase (MAPK) activity. Therefore, we investigated whether KS28 regulated c-fos and AP-1 transcriptional activity in A375R cells. The results showed that the transcriptional activity of c-fos and AP-1 was slightly inhibited by vemurafenib in A375R, whereas these transcriptional activities could be significantly inhibited by KS28. The results suggested that the inhibition of c-fos and AP-1 promoter activity is one of the mechanisms on the anti-proliferative effect of KS28 in vemurafenib resistant cells.

Figure 8

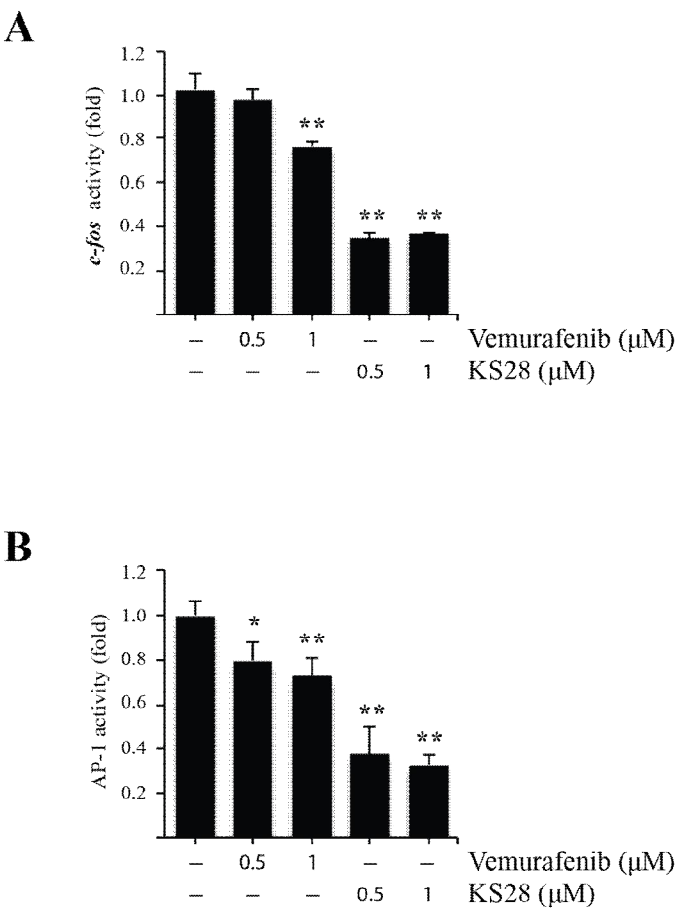

Figure 8. Inhibitory effects of KS28 on *c-fos* and AP-1 promoter activity in A375R cells

(A-B) Cells were seeded and transfected with the luciferase reporters, *c-fos-luc* (A) and, AP-1-luc (B) promoter gene, respectively, with the pRL-TK vector. After transfection for 24h, cells were treated with indicated doses of vemurafenib and KS28 for 24h and harvested. In all of the promoter assays, the firefly luciferase activity was determined in cell lysates and normalized against renilla luciferase activity, and these luciferase activities are expressed relative to control cells. The results represent means ± S.D. standard deviations of triplicate experiments compared with control cells (*p<0.05; **P<0.01).

9. KS28 suppresses anchorage-independent growth of vemurafenib resistant cells

The AP-1 as a transcription factor that mediates responsiveness to tumor promoters, and is composed of the Jun and Fos oncoproteins, involved in growth control and oncogenesis [19] [20]. Therefore, we performed soft agar assays to confirm KS28 can suppress anchorage-independent cell growth by inhibiting MAPK signaling and AP-1 activity in vemurafenib resistant A375R cells. A375 and A375R cells were treated with indicated concentrations of vemurafenib or KS28 in a soft agar matrix and incubated at 37°C in a 5% CO2 incubator for 10 days. Representative images demonstrate a profound reduction of the colony formation mediated by the treatment of KS28 compared with untreated control group (Figure 9). The results showed that the treatment of vemurafenib in A375 cells suppressed colony formation, whereas the treatment of vemurafenib in A375R cells did not cause colony inhibition. And KS28 significantly suppressed the colony formation of A375R cells compared with vemurafenib in A375R cells. These data strongly indicated that KS28 plays an inhibitory role of tumorgenesis in vemurafenib resistant cells.

Figure 9

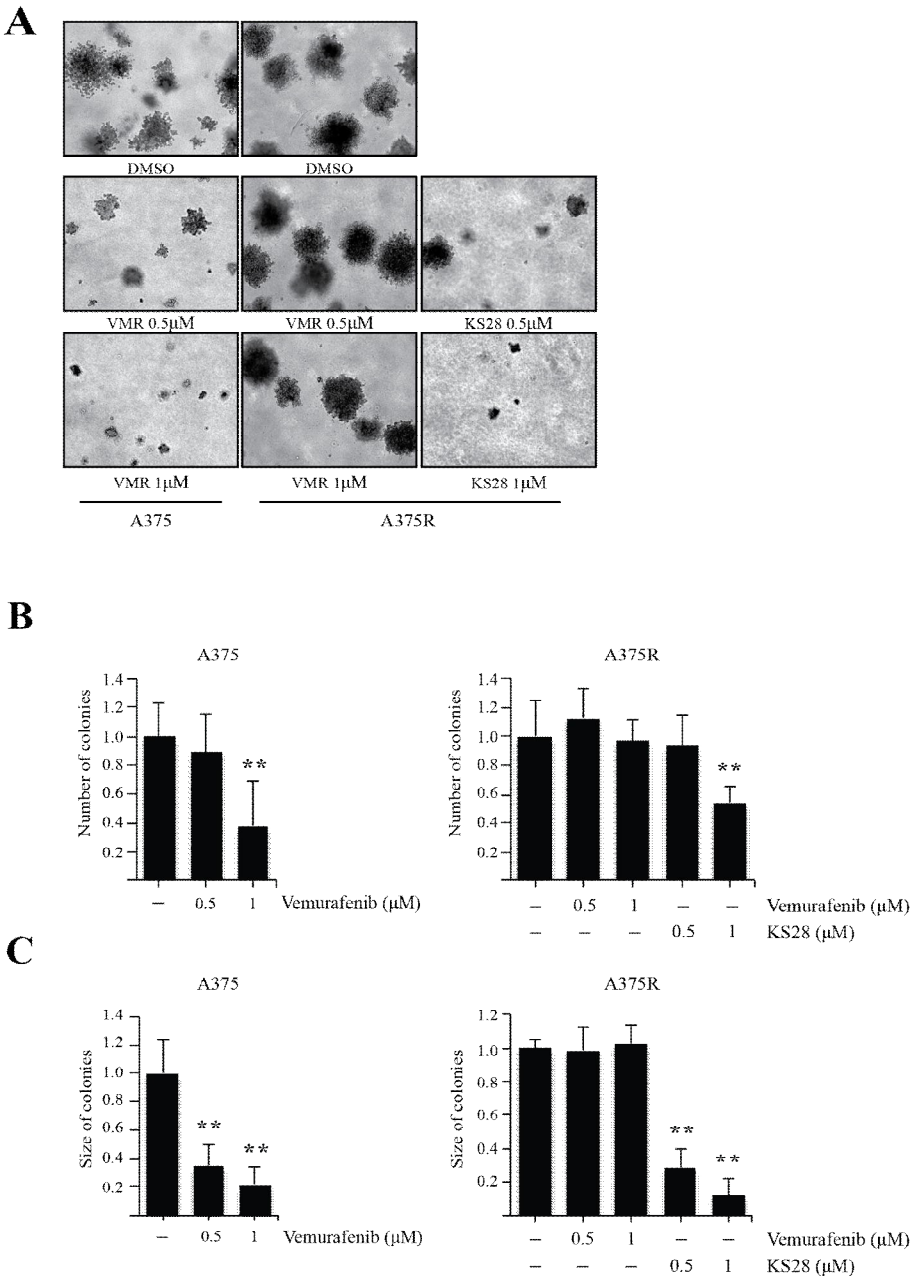

Figure 9. Inhibitory effects of KS28 on tumorigenicity of A375R cells

A375 and A375R cells were exposed to indicate concentration of vemurafenib and KS28 in soft agar matrix and incubated at 37°C in 5% CO_2 atmosphere for 10 days. The representative colonies from three separate experiments were photographed (A) and average number of colonies and sizes were measured under a microscope with the aid of the Image-Pro Plus software program (B, C).

IV. Discussion

Vemurafenib (PLX4032), a selective and potent inhibitor of mutated BRAF, has demonstrated antitumor efficacy in metastatic melanoma, resulting in approximately a 50% response rate and an 80% disease control rate [21]. However, the clinical response to vemurafenib observed to date is relatively short-lived, followed by relapse and resistance [22]. Therefore, acquired drug resistance has become an important issue, and many basic studies are currently underway. Initial studies in several groups have shown that the MAPK pathway is reactivated in resistant tumors [23]. In this study, we examined the novel compound KS28 as a RAF inhibitor for overcoming vemurafenib resistance by regulating MAPK pathway reactivation.

One of the most common intracellular signaling pathway cascades regulated by RTKs is the mitogen-activated protein kinase (MAPK) cascade [24]. This cascade initiates with RAS activation which is bound to GDP in inactive state and becomes activated when binding to GTP. GTP-bind RAS activates the first serine-threonine kinase (RAF), and then RAF directly activates MEK1/2 by phosphorylating multiple serine residues. Consecutively, ERK1/2 is activated by phosphorylating threonine and tyrosine residues [25]. The activated ERK translocates into the nucleus and activates transcription factors through phosphorylation [26]. Several downstream of ERK, such as other protein kinases, gene regulatory protein, and transcription factors, are phosphorylated by ERK and then functions a variety of physiological and pathological responses [27]. Unlike the narrow substrate specificity of RAF and MEK, since ERK has numerous downstream cytoplasmic and nuclear substrates, it can also activate RSK1 (p90 ribosomal S6 kinase 1) in cytosol [28]. RSK1 translocates to the nucleus and activates c-Fos [29]. In this study, KS28 exhibited a strong inhibitory effect on the signaling pathway of MEK, ERK, p90RSK, and c-Fos in JB6 Cl41 cells induced by EGF. Furthermore, it inhibited the phosphorylation of MEK, ERK, p90RSK, and c-Fos

in vemurafenib resistant cells. These results indicated that KS28 could inhibit MAPK pathway signaling and that inhibition of the MAPK pathway could affect the suppression of neoplastic transformation in JB6 Cl41 cells and overcoming vemurafenib resistance.

The c-Jun N-terminal kinase (JNK) pathway, one of the major signaling cassettes of the MAPK signaling pathway, also plays an important role in apoptosis, inflammation, cytokine production, and metabolism [30]. They are activated in a similar way to ERK, but the upstream activator proteins are different. Instead of RAF, RAS activates RAC (MAPKKK), which activates MKK4/7 (MAPKK) and then activates JNK1/2 [31]. Immediately after activation, JNKs translocate to the nucleus and is usually activated in physical association with target transcription factors such as c-Jun, ATF, and Elk1. And then, activated c-Fos and c-Jun heterodimerize to form a more stable AP-1 (activator protein 1) dimer complex, which regulates gene expression in response to various stimuli [32]. In this study, KS28 inhibited EGF-induced phosphorylation of JNK and c-Jun in JB6 Cl41 cells. In addition, KS28 inhibited the EGF-induced c-fos and c-jun promoter activity. This led to the suppression of AP-1 promoter activity. These results indicated that inhibition of JNK pathway is one of the mechanisms on the anti-proliferative effect of KS28.

For resistance occurring after vemurafenib treatment, clinical trials have begun to test the combination or succession of therapies in patients with melanoma who are already at high risk or have advanced recurrence [13] [33]. Inhibition of MEK and mutant BRAF kinase could be a strategy to obtain a more durable response than the inhibition of BRAF alone [14]. That is, combination therapy with other molecular targeting agents could increase the therapeutic efficacy of cancer. However, resistance to combination therapy may also occur. For example, despite the clinical advantages of BRAF or MEK inhibitors, the emergence of resistance to BRAF inhibitors and MEK inhibitors limits the therapeutic efficacy of these kinase inhibitors [34]. In addition, it is poorly understood whether MEK inhibitors are effective after BRAF inhibitor resistance has already developed [35]. Therefore, it is necessary to carefully study the administration, timing, and

side effects of combination therapy to overcome drug resistance in melanoma. In recent studies, to overcome vemurafenib resistance, more potential molecules targeting other aberrant signal pathways are under clinical development [33]. In this study, we confirmed that the inhibitory effects of KS28, a novel compound, on cell viability of vemurafenib resistant cells are more effective than combination therapy. Together these results suggested that KS28 can contribute to the treatment of melanoma resistance. However, since no clinical trials have been conducted, further studies need to be explored to see if they are clinically efficacious.

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ABSTRACT

The mechanistic study of a novel compound on the inhibition of neoplastic cell transformation and vemurafenib resistance

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Melanoma is cancer caused by malignization of melanocytes and occurs mainly in the skin. It is the most dangerous type of cancer that progresses very fast and the mortality rate has recently increased. Most patients with metastatic melanoma harbor BRAF V600E mutation. Thus, to treat this, vemurafenib, the most representative and specific inhibitor of BRAF V600E has been developed and widely used. However, continuous administration of vemurafenib causes reactivation of MAPK, which makes it easy to develop drug resistance. Therefore, the acquired resistance to vemurafenib has become an important issue. To overcome this issue, it is necessary to understand and study various molecular mechanisms and also, we need to develop new treatment strategies and effective new compounds for overcoming vemurafenib resistance.

In this study, we investigated antitumorigenic effects and molecular mechanism of KS28, a novel compound developed as a derivative of vemurafenib, on the inhibition of EGF-induced cell transformation in epithelial cells. And we also investigated inhibitory effects in vemurafenib resistant cells. First of all, we studied antitumorigenic effects of KS28 in epithelial cells. This compound inhibited the cell viability and proliferation of JB6 Cl41 cells and also phosphorylation

of MAPK, c-Fos, JNK, and c-Jun induced by epidermal growth factor (EGF). Moreover, KS28 inhibited EGF-induced c-Fos, c-Jun, and AP-1 promoter activity and suppressed cell transformation of JB6 Cl41 cells in a dose-dependent manner. These results indicated that the inhibition of MAPK and JNK pathway by KS28 might be responsible for KS28`s strong inhibition of neoplastic transformation.

And then, to confirm the inhibitory effects of KS28 on resistance to vemurafenib in melanoma cells, we established vemurafenib resistant cells by continuous exposure of vemurafenib to melanoma cells. We confirmed that MAPK reactivation and expression of c-Fos, JNK and c-Jun were increased in these resistant cells. As a way to overcome resistance, recent studies have reported that combined inhibition with vemurafenib and other molecular targeting agents such as MEK inhibitors may increase the therapeutic efficacy in resistant cells. In this study, treatment with KS28 alone was found to be more effective than the combination of vemurafenib and MEK inhibitor (PD98059) on inhibiting cell viability and phosphorylation of ERK in vemurafenib resistant cells. Thus, this new compound could confirm its potential as a therapeutic agent to overcome vemurafenib resistance. After investigating the molecular mechanism, we found that this compound inhibited the reactivation of MAPK, phosphorylation of c-Fos and also inhibited c-Fos and AP-1 promoter activity dose-dependently. In addition, it was observed that KS28 can suppress tumorigenicity of A375R in soft agar matrix.

Taken together, these results suggest that KS28 could overcome vemurafenib resistance by blocking MAPK signaling pathways and suggest the potential as a novel compound.

감사의 글

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To Poshan, my best buddy and senior. Thank you for always being kind to me and support so much. I'll keep all the memories with you. And I hope that your work will be finished well. You can do very well.

To Muna, also my best buddy. I'm sorry I didn't teach you much. But I think you can overcome everything because you're really strong and outstanding. I'm glad you're adapting to Korea. Let's talk in Korean language later.

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