Farnesyltransferase Inhibitors; Mechanism of Action through Regulation in Cell Cycle Distribution and Ras Effector Molecules

Thesis by
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Farnesyltransferase Inhibitors; Mechanism of Action through Regulation in Cell Cycle Distribution and Ras Effector Molecules

Directed by Professor Si Young Song

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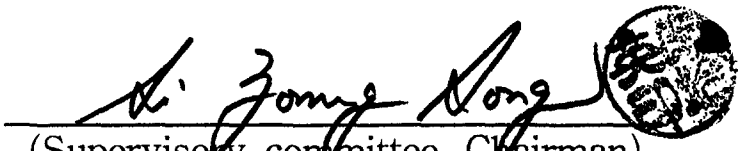

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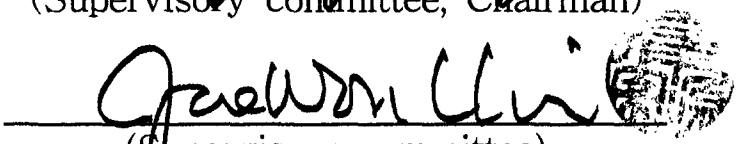

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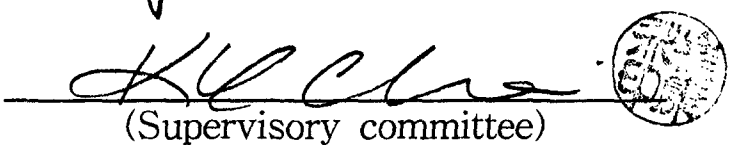

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감사의 글

먼저, 변함없이 저와 함께 하시고 좋은 것으로 채워주시며 지켜주시는 하나님께 감사와 영광을 드립니다. 항상 부족한 저를 따뜻한 격려와 관심으로 참다운 과학자의 길을 보여주신 송시영 교수님께 감사를 드립니다. 이 논문이 있기까지 여러 가지 조언과 지도를 아끼지 않으신 최강열 교수님과 최재원 교수님, 그리고 바쁘신 와중에도 실험하는데 용기를 북돋아 주신 김호근 선생님께 감사드립니다. 또한, 곁에서 지도해 주신 박승우 선생님과 김태일 선생님 그리고 백용한 선생님께 감사의 마음 드립니다.

곁에서 동반자로서 항상 허물없이 편하게 대해준 지은언니, 우리방의 살림을 도맡아 하느라 수고하는 경화언니, 실험으로 바쁘지만 여러 가지로 조언을 아끼지 않으신 이정희 선생님, 열띤 토론을 통해 실험에 도움을 준 선아언니, 힘들지만 꿋꿋이 연구에 몰두하는 지영언니와 재정이, 모두에게 감사의 말씀을 전합니다. 연구 진행에 "도우미" 역할을 해주신 정효영 선생님과 이광형 선생님, 실험실의 왕언니 역할을 해주시는 진수현 선생님과 열심히 실험에 임하는 양순, 지숙 그리고 작은 정희 에게도 고마운 마음을 전합니다. 힘들때마다 곁에서 든든한 후원자가 되어준 윤희언니와 나의 동기 승진이, 항상 명랑한 웃음으로 반겨주던 명진언니 그리고 정진언니, 큰언니같이 든든한 은정언니, 나의 단짝 민정이, 무엇이든 자상하게 가르쳐주신 박기숙 선생님, 친오빠 이상으로 대해주신 김선홍 선생님과 김남균 선생님 그리고 병진오빠, 의욕을 북돋아준 혜진언니, 항상 다정하게 대해 준 은송이와 현정언니 그리고 은숙언니, 힘든일도 마다하지 않고 도와준 약리학 교실에 정연언니, 따뜻한 마음으로 위로가 되어준 인숙언니 그리고 지영언니, 늘 밝은 미소로 대해준 주영언니와 진아, 모두 내겐 잊을 수 없는 사람들입니다. 또한 FACS analysis를 도와주신 길미화 선생님께 더불어 감사 드립니다.

먼 곳에서 항상 응원해주는 나의 가장 소중한 친구 윤지, 나의 생각을 가장 잘 알아주는 유경이, 나에게 아낌없이 주는 해뜨리, 부족한 언니를 신뢰로 지켜봐준 하나뿐인 동생 지원이와 작은 것 까지도 챙겨주시고 애정과 관심으로 대해주시는 종순 아줌마 그리고 아저씨, 늘 변함없이 곁에 있어준 영원한 친구 상우에게도 고마움을 전합니다. 마지막으로, 항상 늦은 귀가 시간까지 저를 기다리시고, 기도와 크신 사랑으로 항상 보살펴주시는 가장 소중한 부모님께 사랑한다는 말을 전하며 이 논문을 바칩니다.

2000년 12월
김주원

Contents

List of Figures

Abstract

Farnesyltransferase Inhibitors: Mechanism of Action through Regulation in Cell Cycle Distribution and Ras Effector Molecules

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(Directed by Professor Si Young Song)

Farnesyl transferase inhibitors (FTIs) are novel class of antitumor drugs that block the oncogenic activity of Ras by blocking the farnesyl protein transferase (FPTase) that catalyzes the first of a series of posttranslational modifications of Ras required for full biological activity. Novel FTI by LG, LB42908, and peptidomimetic FTI by Merck, L744,832, were used in the present study to elucidate growth inhibitory effect of FTIs in cells transfected with activated H-Ras (H-Ras RIE-1 cells) or K-Ras (K-Ras RIE-1 cells) cDNAs in RIE-1 cells. Furthermore, the mechanism of which the FTIs suppress cell proliferation was investigated. The FTIs selectively inhibited growth of H-Ras RIE-1 cells whereas the control cells were unaffected. LB42908 inhibited cell growth in all three cell lines, however, H-Ras RIE-1 cells were growth inhibited at a lower dose than that of the other cell lines.

According to the FACS analysis, it was determined that FTI treated H-Ras RIE-1 cells accumulated at a G1 phase in an expense of the S phase at a concentration of 10 μM while K-Ras RIE-1 cells and control cells displayed little change in cell cycle distribution. Western blot analysis showed that both FTIs resulted in reduction of cyclin A, cyclin D1 and MAPK activities. While the cyclin D1 expression was decreased in both the H-Ras and K-Ras transfected cells, near-complete reduction in the level of cyclin A and MAPK activity was observed in H-Ras RIE-1 cells, which correlates with the results from FACS analysis and MTT assay, where the H-Ras showed more sensitivity toward FTIs. However, PI 3-kinase, a downstream effector molecule of Ras, was not affected by both FTIs at a dose that inhibited MAPK activity, cyclin D1, and cyclin A expression. The results demonstrate that the cyclin A and MAPK play important roles in selectivity to H-Ras transformed cells, whereas the cyclin D1 and AKT plays a lesser role. In conclusion, FPTase inhibitors are potent anti-tumor agents that inhibits cell growth by inhibiting G1-S cell cycle progression through regulation of Ras effector molecules.

Key Words : Ras, FPTase Inhibitor (FTI), MAPK, Cell Cycle
Cyclin D1, Cyclin A

Farnesyltransferase Inhibitors: Mechanism of Action through Regulation in Cell Cycle Distribution and Ras Effector Molecules

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The Graduate School, Yonsei University

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I . Introduction

Over the century, tremendous efforts have been made to unveil the genetic and biochemical mechanisms underlying the pathogenesis of the cancerous phenotypes. Viral *ras* genes were among the first oncogenes isolated and cloned.[1] Subsequently, mutated forms of cellular Ras genes are frequently found in >90% of pancreatic and >50% of colon adenocarcinomas.[2] By studying the biology and biochemistry of Ras protein, it was found that the Ras protein plays a significant role as a membrane bound GTP binding protein that serves as molecular switch in mitogenic signal transduction.[3] Ras protein normally responds to growth stimuli by exchanging the GDP bound form for the GTP bound form, which successfully activates mitogenic activating protein kinase (MAPK), downstream of Ras, within a few minutes and induces cell

proliferation. This signal transduction is terminated when the GTP bound Ras protein is dephosphorylated to the GDP bound form by GTPase.[1,4] Constitutive activation of receptor tyrosine kinases can also be oncogenic and may occur via autocrine stimulation by ligand or viral mutations, overexpression, amplification, or post-translational activation of the receptor.[5,6] Furthermore, transformation by activated receptor or nonreceptor tyrosine kinases has been shown to require functional Ras protein. Thus, these observations have suggested multiple targets for pharmaceutical intervention against Ras in mammalian cells.[1,3,7] However, attempts at blocking Ras induced transforming activity with small inhibitor molecule have been unsuccessful. Recently, a novel class of antitumor drugs that can possibly block the oncogenic activity of Ras was discovered by exploiting observations concerning the physiology of Ras protein. Ras protein are modified in a series of reactions that result in either farnesylation or geranylgeranylation at a cystein residue at the fourth amino acid position from the COOH-terminal end. The COOH-terminal CAAX sequence of Ras is recognized by farnesyltransferase when X is methionine or serine and A is isoleucine, leucine, or valine.[8,9] The H-Ras recognition sequence is CVLS, whereas K-Ras A and B have the sequences CVIM and CIIM, respectively.[10] Thus, farnesyltransferase inhibitors (FTIs) which has ability to block farnesylation of the proteins bearing CAAX motif was developed as an antitumor drug.

The FTI, LB42908 synthesized by LG Chemicals, is a nonpeptidic, nonsulfhydryl 1-(1(3)H-imidazole-5(4)-yl)methylpyrrolederivatives that

are potent in enzymatic inhibition of Farnesyl Protein Transferase (FPT) against K-ras, N-ras as well as H-Ras and highly selective over Geranylgeranyl Protein Transferase I (GGPT-1). In an anchorage independent soft agar study, LB42908 effectively inhibited growth of several human tumor cell lines, including HT29, HCT116, A549, EJ, T24. In addition, LB42908 resulted tumor regression in nude mice xenograph without significant weight loss. On the other hand, the FTI developed by Merck Corporation, L744,832, is a peptidomimetic which is a isopropyl ester prodrug derivative. L744,832 also inhibited soft agar colony formation of the human tumor cells lines, and reported to cause G2/M arrest and apoptosis in pancreatic cancer cell lines. The effect of these two peptidomimetic and non-peptidimimetic drugs were compared in this study.

Some of the cascades activated by p21 Ras, such as phosphoinositide 3-OH kinase (PI3K) and the extracellular regulated kinase (ERK) pathways are thought to lead to cell cycle progression and proliferation. The p21 Ras has also been implicated in the activation of p38 mitogen activated protein kinase and Jun N-terminal kinase (JNK), protein involved in the cellular response to stress. ERK activation usually involves participation of Ras and Raf oncoproteins and activation of MEK, a dual specificity kinase that phosphorylates ERK1 and ERK2.[11] Activation of AKT/Protein Kinase B, which lies down stream of PI3K via Ras activation also promotes cell proliferation, and several lines of evidences suggests that the PI3K and AKT is required for full transformation phenotype by Ras.[12,13] AKT, a subfamily of

serine/threonine protein kinase, has been identified as a direct target of PI3-K. Furthermore, Ras has been implicated in the positive regulation of the cyclin D1 promoter, and activated form of the MAPK kinase, MEK, reproduce the effect of oncogenic Ras on cyclin D1.[14]

Pharmacological inhibition of FTase has been shown to inhibit Ras in both H- and K-Ras transformed cells, although in most cell types, K-Ras is far less sensitive.[15] Resistance of K-Ras to FTIs may occur because its compensatory geranylgeranylation by GGPTase I.[16] Unexpectedly, FTIs have no apparent growth inhibitory effect on normal cells, and inhibition of cells by FTIs does not always correlate with Ras mutational status.[17] In other words, the FTIs are able to specifically inhibit tumors bearing Ras mutation. In pancreatic cancer cell lines, FTIs have been reported to induce apoptosis by inhibition of growth factor induced and integrin mediated AKT2 activation and AKT2 mediated BAD phosphorylation.[18] In MCF7 cell line, cell proliferation was inhibited by FTI accompanied by G1 arrest and hyperphosphorylation of pRb.[19] It was also shown that the FTI inhibited farnesylation generally and H-Ras processing specifically. Accordingly, FTIs effectively revert the phenotype of H-Ras transformed fibroblast and have remarkable effects on animal tumors that harbor H-Ras mutation.[20] However, the different mechanism of action of FTI in different Ras types have not been completely explained.

The mechanism underlying FTI action is interesting because its action is likely to be secondary to specific inhibition of farnesyltransferase, which suggests that several farnesylated protein yet

to be identified play a critical role in the biology of transformation.[19] Furthermore, exact mechanism for their inhibition of cell growth needs to be further elucidated.

Hence, the present study aimed to elucidate the mechanism by which FTI arrests the growth of the cancer cells and the biological basis of mechanism for their inhibition of cell growth. In addition, the drug selectivity of FTIs in two different Ras isotypes, H-Ras and K-Ras, were tested with two different FTIs, LB42908 and L744,832. First of all, b growth inhibitory effects of FTIs used in this study was determine by MTT assay, and FTIs were most sensitive in H-Ras overexpressed rat intestinal epithelial (H-Ras RIE-1) cells. Evaluation of MAPK expression in H- and K-ras transfected RIE-1 cells showed decrease in ERK activity after treating the cells with FTIs, while no apparent change in ERK activity was observed in control cells. The FACS analysis showed accumulation of G1 cells, that is, G1 arrest in H-Ras RIE-1 cells and K-Ras RIE-1 cells accompanying reduction of cyclin D1 and cyclin A expression, while no change in cell cycle distribution was observed in control cells (RIE-1 neo) with induction of cyclin D1 activity. Thus, novel FTIs, LB42908, and L744,832, has growth inhibitory effects in cells, especially in those overexpressing H-Ras, and that it shows inhibitory effect through blockage of MAPK pathway and G1 cell cycle arrest followed by reduction in cyclin D1 and cyclin A activity while no apparent change was observed in CDK4 and CDK6.

Ⅱ. Materials and Methods

1. Cells and Culture conditions

RIE-1 cell is a diploid, non transformed cell line derived from rat small intestine. RIE-1 cells were stably transfected with control vector construct Neo4F or with constructs encoding activated H- and K-ras4B. The cells were maintained and grown as monolayer cultures in Dulbeccos Modified Eagles Medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 2.5 g/ml amphotericin B, and 10% fetal bovine serum (FBS)(Hyclone, Logan, UT, USA) at 37℃ in humidified atmosphere of 5% CO_2 in air.

2. Farnesyltransferase inhibitors

L744,832 (MW=558.78) and LB42908 (MW=605) were obtained from Merck Research Laboratories (West point, PA, USA) and LG chemicals (Taejon, Korea), respectively. The structure is noted in Figure 1. L744,832 and LB42908 were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at -20℃.

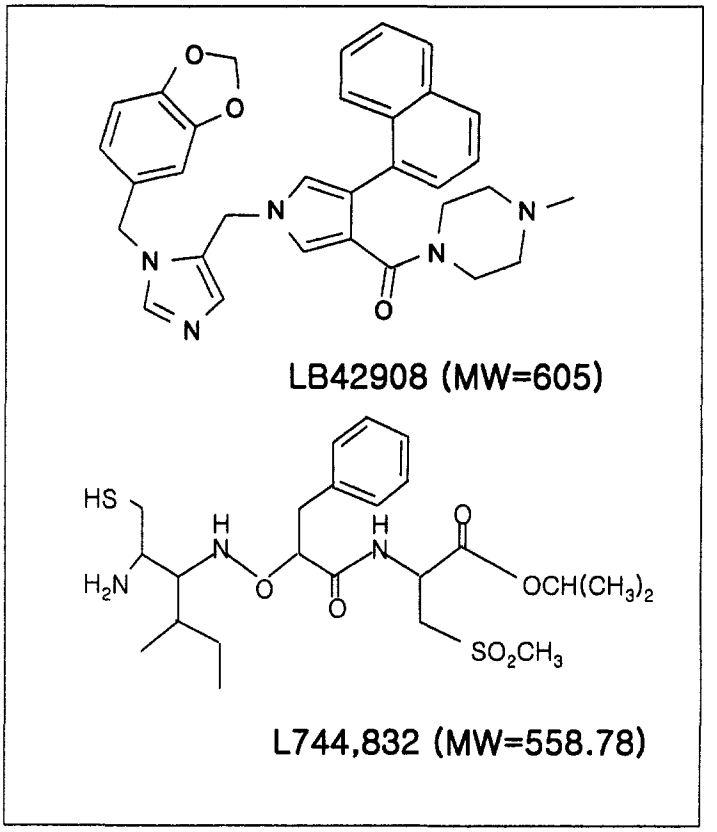


Figure 1. Chemical structure of FTIs, LB42908 and L744,832

3. Transfection

The pZIP-K-ras(12V) retrovirus expression vector construct, which encodes transforming mutants of human K-Ras 4B has been decribed.[21,22] The pSV2-H-ras (12V) expression vector construct contains the genomic human sequences encoding the transforming H-Ras (12V) protein was obtained from Sunnybrook's Health/Science

Center (Toronto, Canada). The constructs pZip-K-ras(12V) with pZIP-NeoSV(x)1, as well as the pZIP-NeoSV(x)1 vector control, were each transfected into the RIE-1 cells (1-3 ㎍ of plasmid DNA/60-㎜ dish). Transfections 16-20 h on cells seeded at 1-5 $\times 10^5$/60-㎜ dish. Transfected cells were selected in medium containing 400 ㎍/㎖ G418 (Life technologies, Rockville, MD, USA). The pSV2-H-Ras (12V) as well as the pSV2-neo vector control were each transfected into the RIE-1 cells by calcium phosphate precipitation. Transfected colonies were selected in medium containing 400 ㎍/㎖ of G418 and subcloned by limiting dilution.

4. MTT Assay

The 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) was performed to determine survival fractions according to the method described. The cells were plated at 2×10^3 cells per well in a 96 microculture well plate. After addition of the various concentrations of FTIs, the cells were incubated for 72 h, and the cytotoxicity of FTI was then measured by the MTT Assay. The plates were washed two times with phosphate buffer saline (PBS). MTT was dissolved at a concentration of 5 mg/ml in sterile PBS and filter sterilized. 50 ㎕ of MTT stock solution was added to each well to be analyzed and incubated for 3 h at 37℃, at which point 100 ㎕ of DMSO was added to each well to dissolve formazan produced. Optical density was measure at 570 nm with a Titer-Tech 96-well multiscanner (Beckton and Dickenson, Heidelberg, Germany) using identically treated wells

containing tissue culture medium alone as the blank.

5. Preparation of Cellular Extracts

Cells were grown to 70% confluence, washed twice with PBS, then switched to 10% FBS contained DMEM with 10 μM of either vehicle or LB42908/L744,832 and then incubated for 48 h. The cells were washed with PBS twice then trypsinized with 0.25% trypsin-EDTA (GibcoBRL, Grand Island, NY, USA) and collected in a 15 ml conical tube with DMEM containing 10% FBS. The tubes were centrifuged at 1500 g for 5 min. The cells were washed with PBS and collected in a 1.5 ml eppendorf tube and then quickly centrifuged. Cell pellets were resuspended with protein lysis buffer containing 1M β-glycerophosphate (pH 7.2), 50 mM sodium vanadate, 0.5 M $MgCl_2$, 0.2 M Ethylene Glycol-bis(-Aminoethylether)-N,N,N',N'-Tetraacetic acid (EGTA), 1 M DTT, 100% Triton, 100 mM Phenylmethyl Sulfonyl Fluoride (PMSF), and protease inhibitors (a cocktail solution with 5 mg/ml aprotinine, leupeptine, pepstatin, and antipain) and incubated on ice for 1 h then centrifuged at 13000 g for 15 min. The supernatant was collected in ice cold 1.5 ml eppendorf tube and stored at -70℃ for further analysis.

6. Fluorescence Activated Cell Sorting Analysis

Cells were seeded onto polyHEMA coated dishes in the presence or absence of FTI unless otherwise indicated. After 48 h, cells were harvested and washed twice with PBS and fixed in 100% ethanol

overnight at -4 ℃. The cells were then centrifuged at 1500 g for 5 min, and cell pellets were washed with 1 ml of PBS. After centrifugation, cell pellets were resuspended with 500 ㎕ of PBS containing 10 U/ml RNase A for hydrolysis, and then 100 ㎍/ml propidium iodide was added to each sample tubes. FACS ananlysis was done essentially for cell cycle analysis (Beckton and Dickenson, Heidelberg, Germany). The cell cycle distribution was determined using ModiFit LT Software (Verity Software House, Popsham, ME). All experiment was repeated at least two times.

7. Western Blot Analysis

Protein concentration was determine by using Bradford Method (Bio Rad, Hercules, CA, USA) and bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used as the standard. 50 ㎍ of protein were separated by 10% SDS polyacrylamide gel electrophoresis (PAGE), and transfered to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA, USA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). Antibodies used were as follows: p-ERK (mouse monoclonal, Santa Cruz Biotechnology), ERK (mouse monoclonal, Santa Cruz Biotechnology), p-AKT (rabbit polyclonal, New England Biolabs, Beverly, MA, USA) which specifically detects phosphorylation site at serine 473, cyclin D1 and cyclin A (mouse monoclonal, Santa Cruz Biotechnology), CDK4 and CDK6 (rabbit monoclonal, Santa Cruz Biotechnology) and H-RAS, K-RAS, and Pan-RAS (mouse monoclonal, Santa Cruz Biotechnology).

The primary antibodies were diluted in a 1 to 1000 ratio with Tris-buffered saline with 0.05% tween-20 (TBST), and incubated for 1h or over night. The secondary antibody was blotted for 45 min, and visualized with enhanced chemoilluminescence solution (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Ⅲ. Results

1. Both FTIs, LB42908 and L744,832 selectively blocks cellular proliferation of H-Ras RIE-1 Cells.

The effect of the farnesyltransferase inhibitors in suppression of the growth of the Ras transformed cells *in vitro* have been proven by several studies done previously.[23,24] It was also established that a peptidomimetic inhibitor of FTase block the anchorge dependent and independent growth of human tumor cell lines.[19] In order to identify the targets responsible for the antiproliferative effects of the FTIs, the effectiveness of two drugs, LB42908 and L744,832, on growth of the parental RIE-1 cells and RIE-1 cells stably transfected with plasmid expressing activate H-ras or K-ras (K-Ras RIE-1 cells) were tested and compared. After transfecting the cells with expressible Ras cDNA contruct, Western blot was carried out to detect the overexpression of p21 H-Ras or K-Ras in RIE-1 cells transfected with H-Ras and K-Ras, respectively. Pan-Ras immunoblotting confirmed the equal amount of total Ras protein in either control cells and transfected cells as illustrated in Figure 2.

The cell growth inhibitory effect of L744,832 and LB42908 was measured by MTT assay as described in Materials and Methods. As a result, L744,832 and LB42908 (from 0.5 μM to 25 μM) selectively inhibited proliferation of activated H-Ras RIE-1 cells as measured by a dose dependent reduction in MTT readings as illustrated in Figure 3 and 4. However, it was determined that L744,832 had no apparent

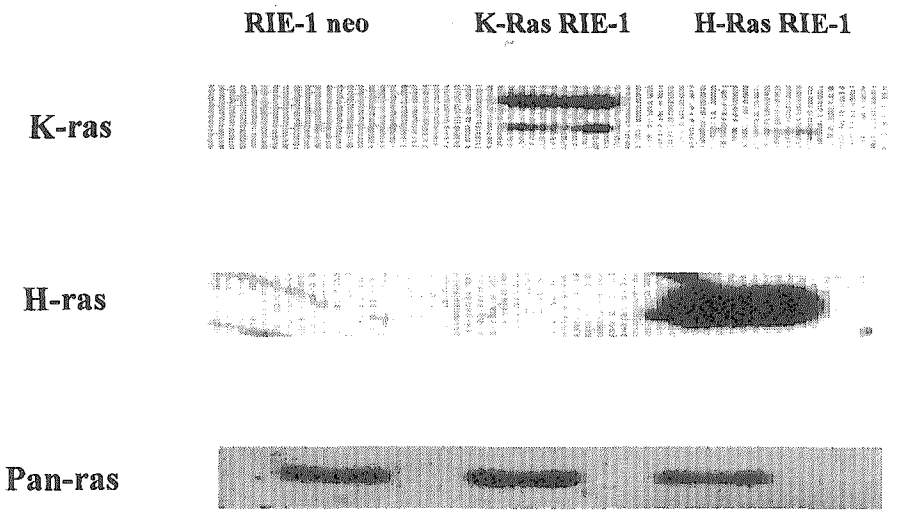


Figure 2. Western blotting to detect overexpression of p21 Ras in transfected cells. Cells were transfected with H- and K-ras showed overexpression of p21 Ras. Pan-Ras antibody used to determine the total Ras protein in control and H- or K- Ras transfected RIE-1 cells.

growth inhibitory effect on normal cells (Figure 4). Also, LB42908 more effectively inhibited cell growth when cells were oncogenically transformed with H-Ras. Finally, both drugs selectively blocked H-Ras RIE-1 cellular proliferation, and LB42908 more effectively inhibited growth of the cells than L744,832.

2. Inhibition of Growth was Accompanied by Cell Cycle Arrest in Cells Treated with FTIs, LB42908 and L744,832.

After investigating the antiproliferative effect of the FTI, the cell cycle status of the cells treated with FTIs was measured. The evidences that the inhibition of farnesylation results the change in cell cycle distribution or in accumulation of cells in G0/G1 or G2/M, depending on the type of human tumor cell lines. This study investigated whether the compounds used causes cell cycle arrest in RIE-1 cells. Cells were treated with vehicle or 10 μM of LB42908 or L744,832 for 48 h as described in Materials and Methods, and their DNA content was analyzed by flow cytometry after staining with propidium iodide. As shown in Figure 5 and 6, FTIs inhibited proliferation of the cells and resulted in an increase in the proportion of G1 cells at the expense of cells in S phase, whereas the proportion of cells in G2 and mitosis remained largely unchanged in H-Ras RIE-1 and K-Ras RIE-1 cells. There was a remarkable increase in G0/G1 fraction, >20% in H-Ras RIE-1 cells and <10% in K-Ras RIE-1 cells.

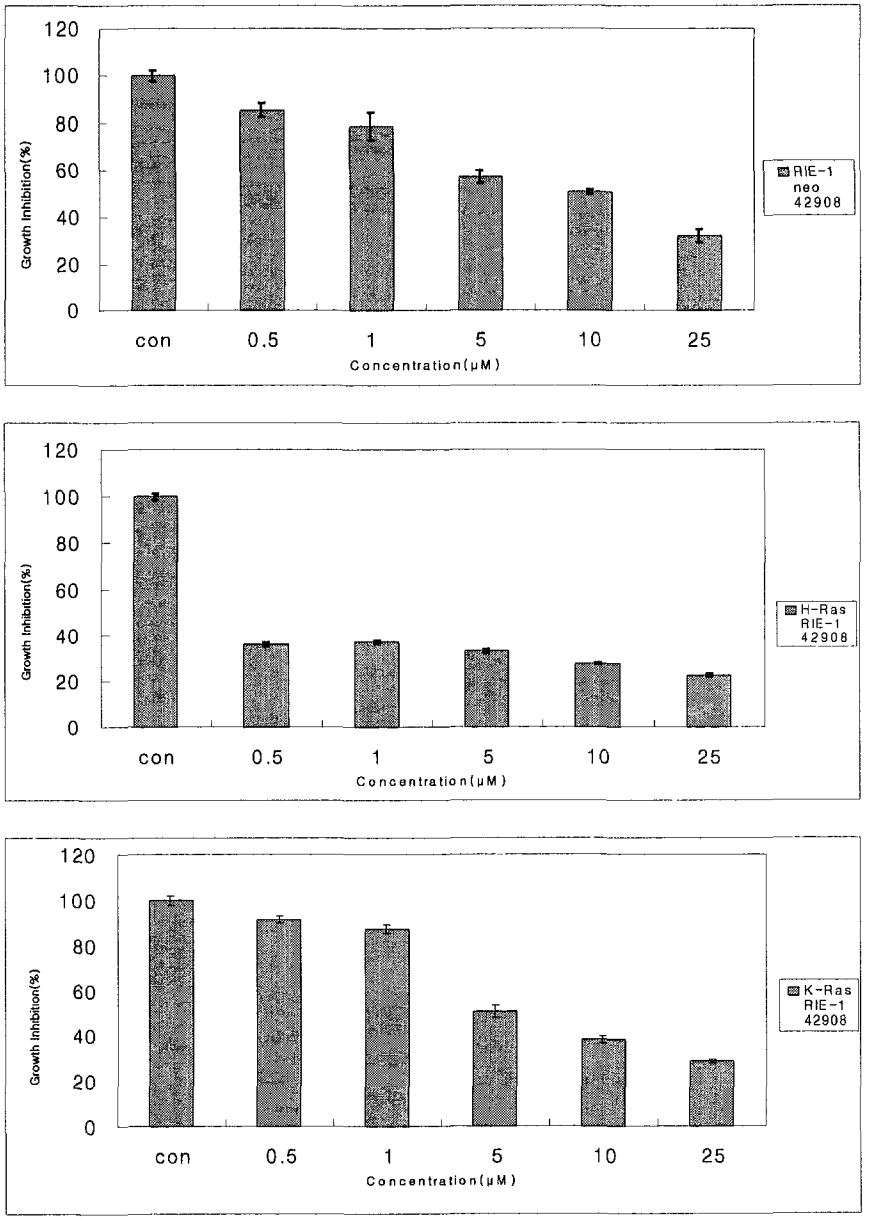


Figure 3. Growth inhibitory effect of LB42908 in H-ras and K-ras transfected RIE-1 cells. Cell viability was measured by using MTT assay following 72 h of LB42908 treatment. Value represent the mean ± SEM from 4 independent experiments.

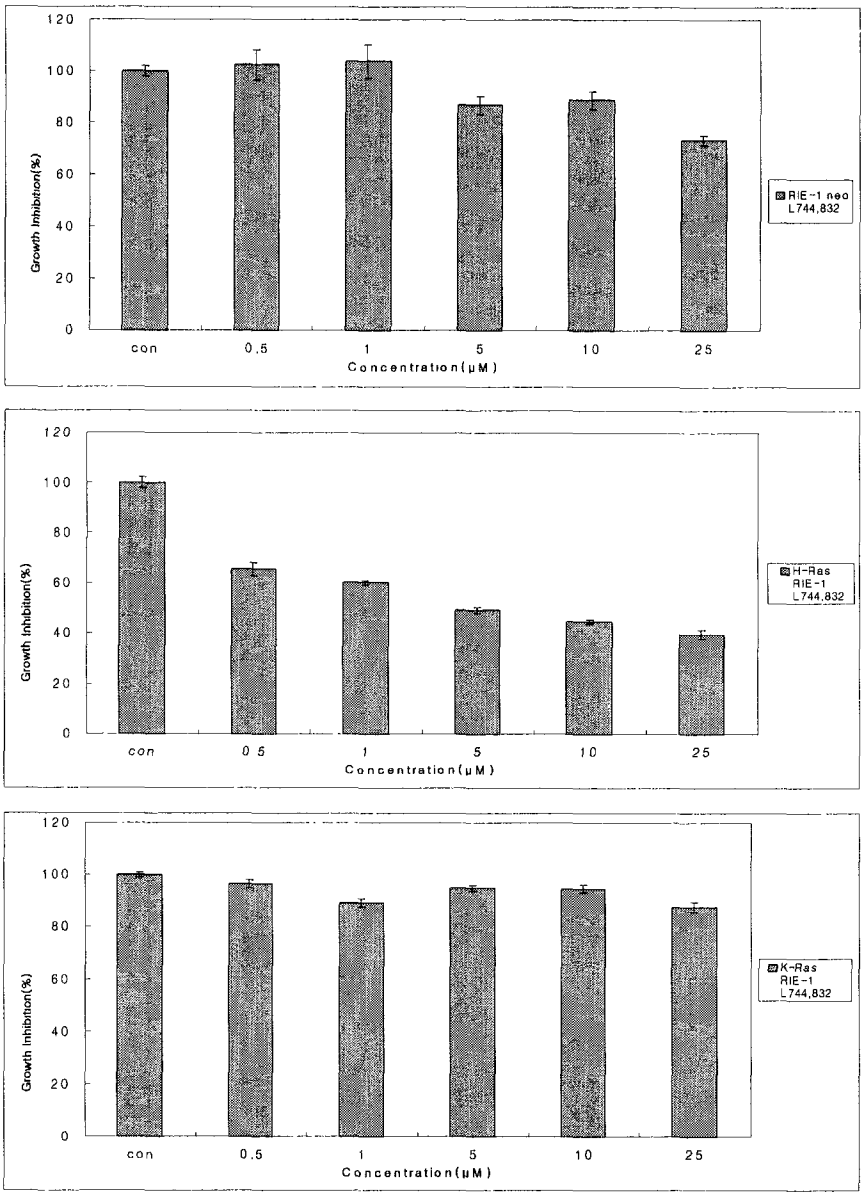


Figure 4. Growth inhibitory effect of L744,832 in H-ras and K-ras transfected RIE-1 cells. Cell viability was measured by using MTT assay following 72 h of L744,832 treatment. Value represent the mean ± SEM from 4 independent experiment.

As expected, the cell cycle distribution of the control cell line, RIE-1 neo was not changed by treatment of FTIs. (Figure 7)

3. Cyclin D1 and cyclin A, a regulator of G1/S transition, was downregulated by FTI in H- and K- ras transfected cells.

Ras has been implicated in the positive regulation of the cyclin D1 promotor, and the conditional expression of oncogenic Ras induces cyclin D1 protein production in growth deprived cells.[25] To elucidate whether FTI which is known to block Ras protein prenylation induces a decrease in cyclin D1 and cyclin A expression, Western blot was carried out. After treating the cells with 10 μM of FTI for 48 h, the cells were harvested and protein was extracted as described in Materials and Methods. Then the protein was subjected to Western blotting with monoclonal cyclin D1 anti-mouse antibody and monoclonal cyclin A anti-rabbit.

As shown in the figure 7 and figure 8, the presence of the LB42908 severely decreased cyclin D1 expression in K- or H-Ras transfected cells whereas the opposite effect was observed in control cells. Similar effect was observed in cells treated with L744,832. The cyclin A expression was decreased in H-Ras RIE-1 cells specifically and slight decrease was observed in K-Ras RIE-1 cells. (Figure 8) However, the levels of CDK4 and CDK6 were unchanged in all three cell lines.

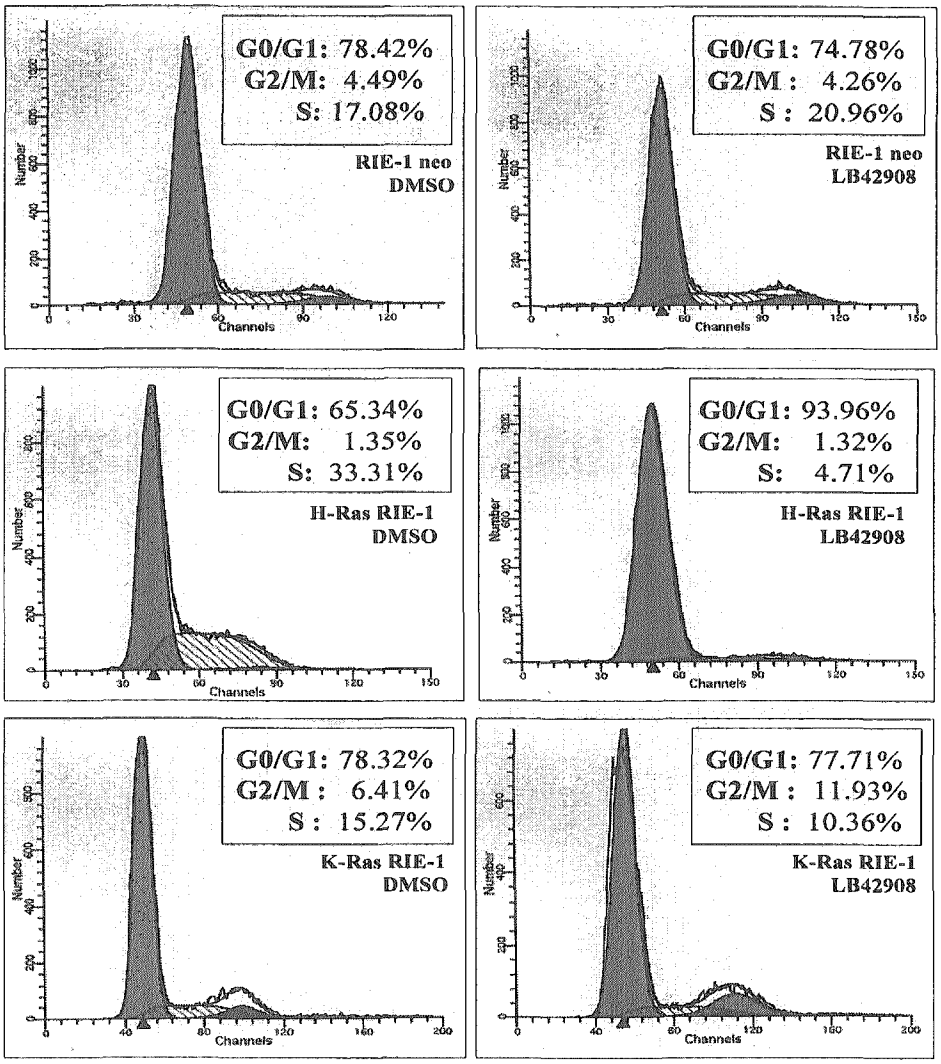


Figure 5. Cell cycle analysis of RIE-1 control cells and Ras transfected cells. LB 42908 (10 μM) blocks G1/S transition in RIE-1 cells. Cells were grown to 30% confluency and treated with inhibitors or vehicle for 48 h as described under "Materials and Methods". Nuclei were prepared by a trypsinization, fixed with ethanol, and DNA were stained with propidium idodide for FACS analysis.

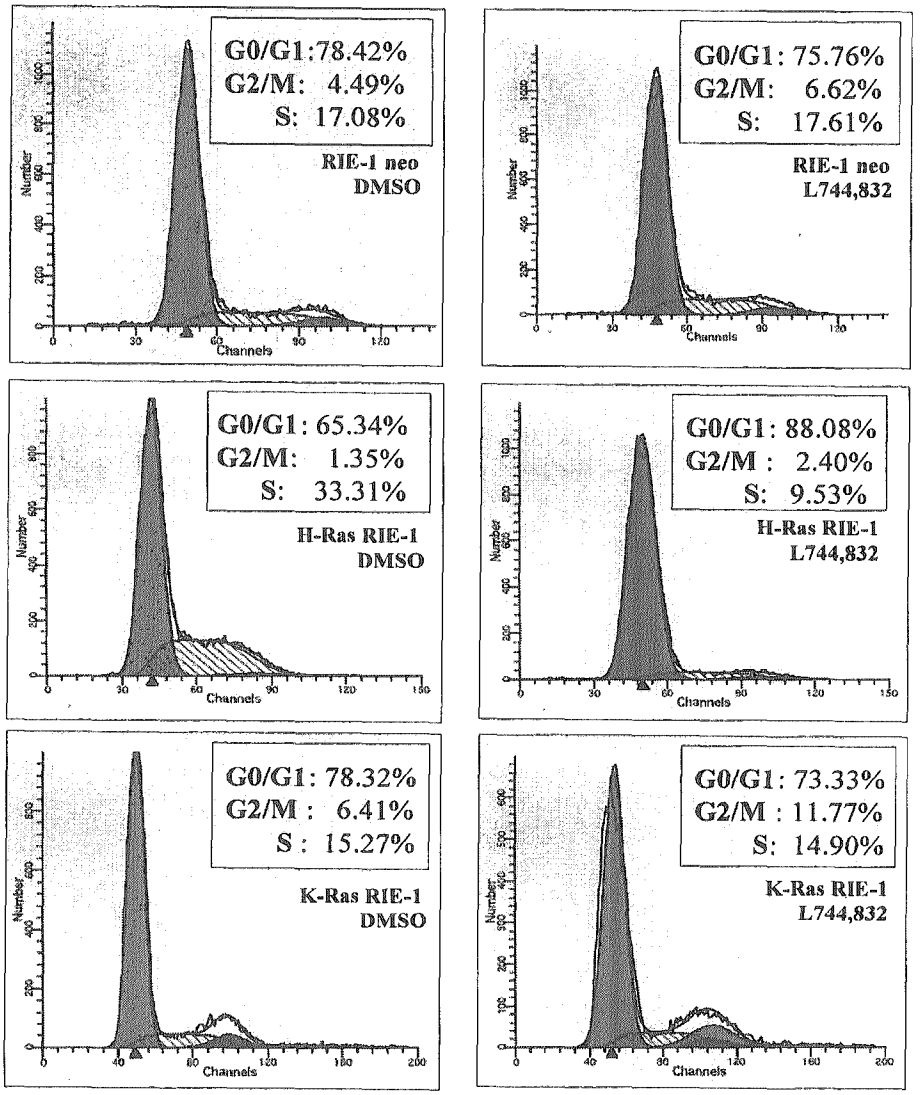
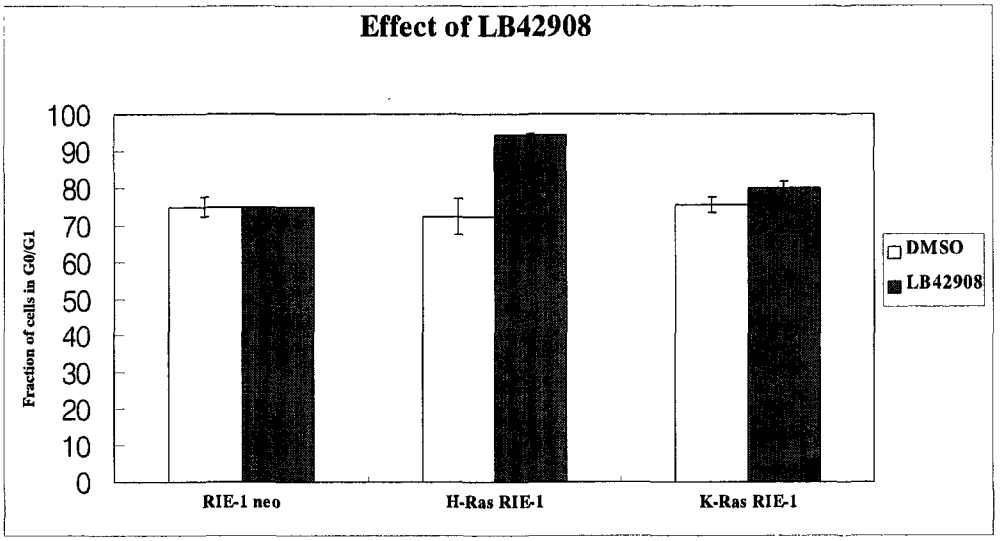


Figure 6. Cell cycle analysis of RIE-1 control cells and Ras transfected cells. L744,832 (10 μM) also blocks G1/S transition in RIE-1 cells. Cells were grown to 30% confluency and treated with inhibitors or vehicle for 48 h as described under "Materials and Methods". Nuclei were prepared by a trypsinization, fixed with ethanol, and DNA were stained with propidium idodide.



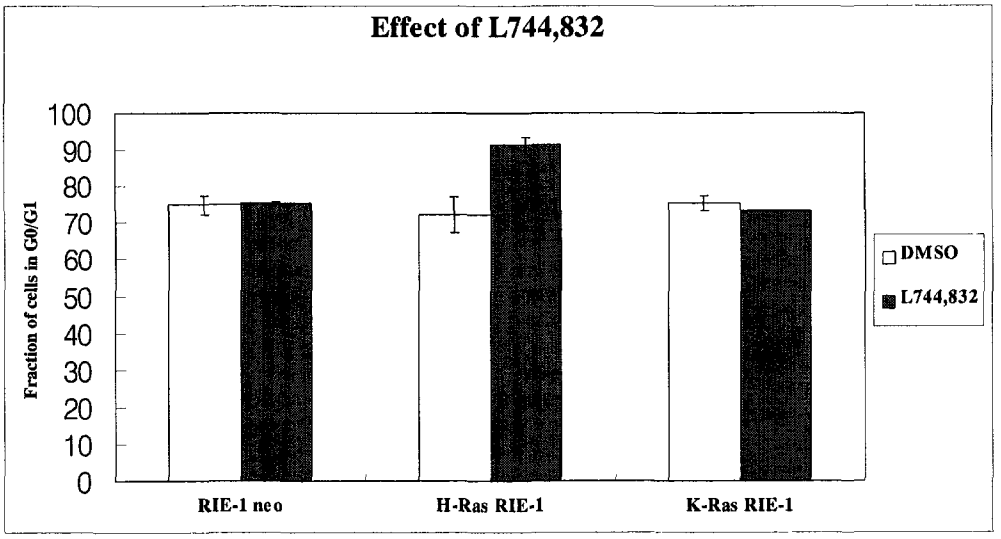


Figure 7. Effect of FTIs on cell cycle distribution in RIE-1 cells. Cells were treated with either L744,832/LB42908 or DMSO vehicle control. Accumulation of G0/G1 fraction of H-Ras RIE-1 cells indicates the blockage of G1/S transition.

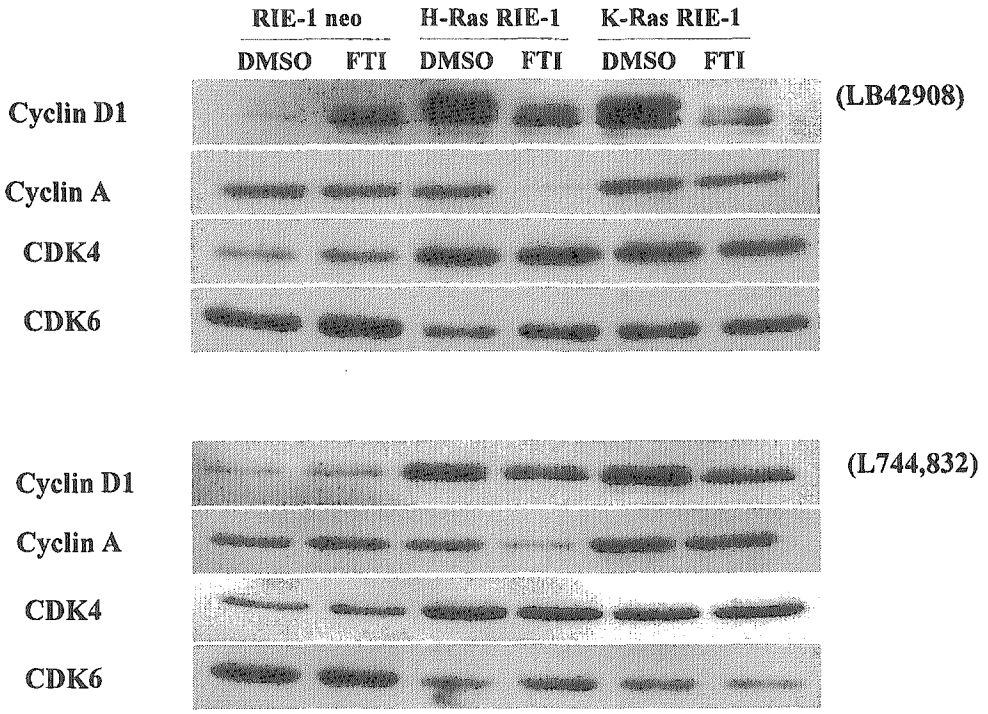


Figure 8. Expression of cyclin D1, cyclin A and CDK4/6 in RIE-1 cells treat with or without FTIs, LB 42908 or L744,832. Both drugs have shown to block cyclin D1 activity in those cells transfected with H- or K-Ras oncogene, where as near complete reduction of cyclin A expression level was observed in H-Ras RIE-1 cells. However, cyclin D1 activity was induced in control cells without Ras overexpression. No change was shown in the levels of CDK4 and CDK6 expression.

4. The ERK Cascade is involved in FTI mechanism of action

Recent findings have shown that the sustained activation of ERK1/2 cascade was required to pass G1 restriction check point entering the S phase in different cell types.[26,27] As illustrated in Figure 9, treating the cells for 48 h with 10 μM of FTIs, L744,832 or LB42908, markedly reduced the ERK 1/2 activities. Moreover, cells transformed with H-Ras oncogene was most sensitive to both drugs, and K-Ras overexpressing cells and control cells show little sensitivity to FTIs. The resistance of K-Ras to such drug may occur because of its compensatory geranylgeranylation by GGPTase I.[16] The total ERK was confirmed by Western botting (Figure 10). This was not due to the toxicity of the drug itself, because both drugs caused morphologic reversion and did not cause apoptosis at a concentration of 10 μM. (Figure 9) Thus, according to results, the FTI has ability to inhibit ERK expression, especially in H-Ras transfected cells.

5. PI3K/AKT signaling pathway was unaffected by both inhibitors

One of the Ras effector molecules, p-AKT (62 kDa) expression was investigated by Western blot analysis in the absence or presence of FTIs. AKT is a Ras effector molecule down stream of PI3K. According to the recent findings, it was reported that PI3K is required for cyclin D1 protein expression and S phase entry in fibroblasts.[14] Figure 11 shows that there are no apparent change in AKT activity levels in all cell lines.

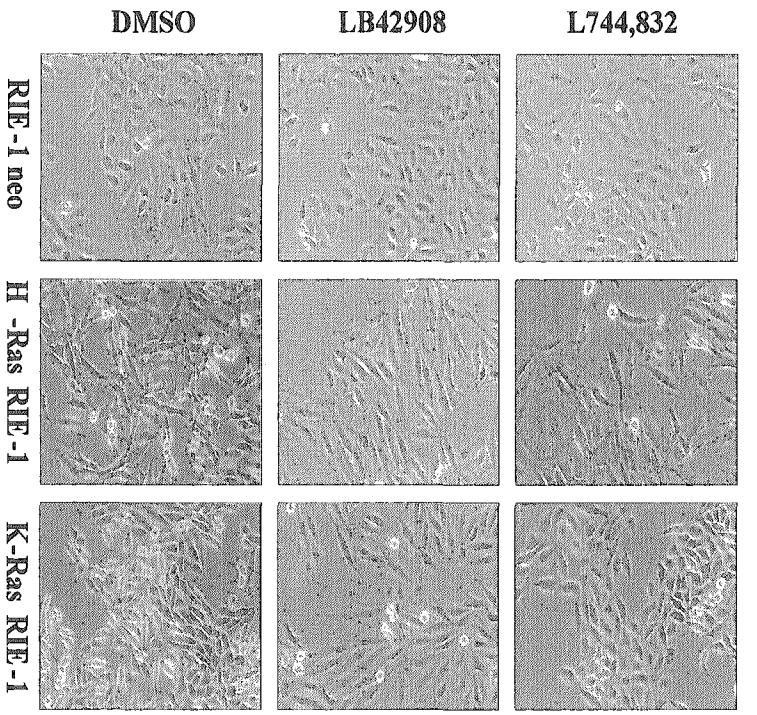


Figure 9. Effects of FTIs (LB42908 and L744,832) on the morphology of RIE-1 cells. RIE-1 cells were treated with DMSO or 10 μM of FTIs. The phase-contrast images were captured at ×400 magnification 48 h after the FTI addition. Six separate experiments were carried out with results similar to those shown here.

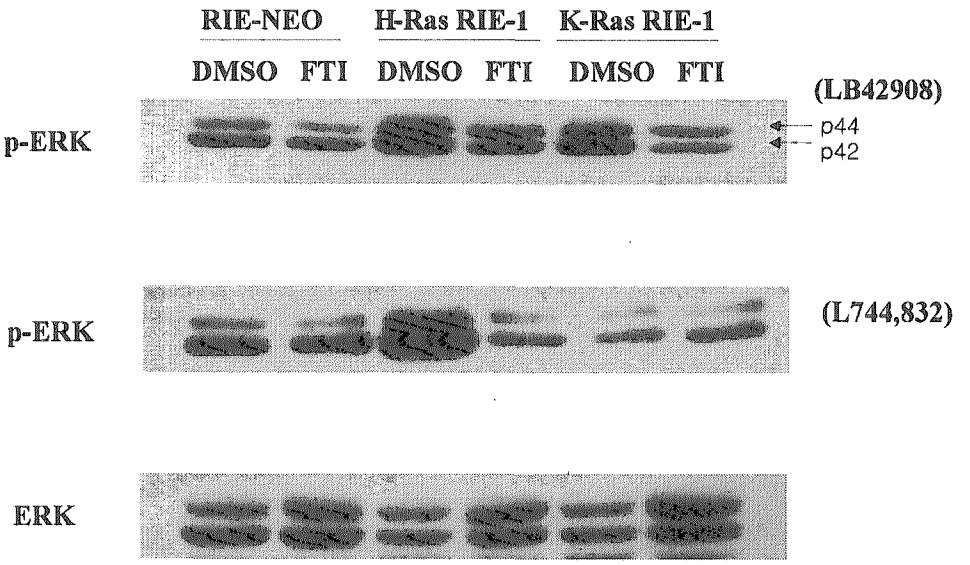


Figure 10. Inhibition of MAPK activity in FTI treated RIE-1 cells. The activity of p-ERK, a molecule down stream of Ras were dowregulated after being treated with 10 μM of FTIs.

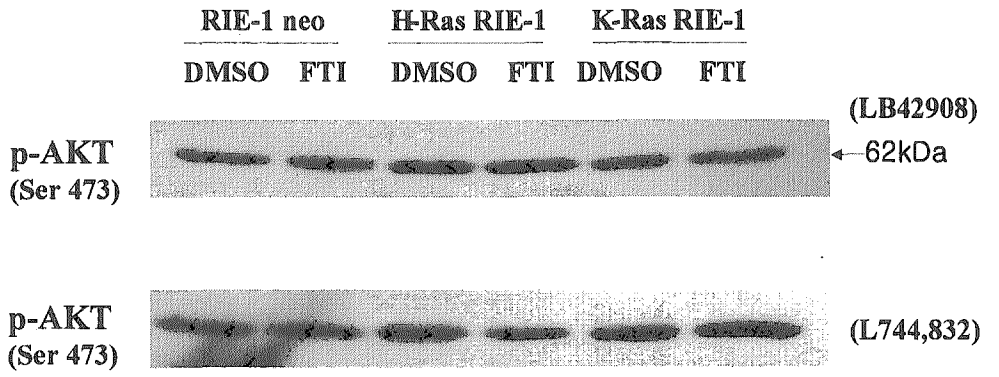


Figure 11. Expression of p-AKT after treating cells with LB 42908 and L744,832 for 48 h. No apparent change was observed in Akt activity in FTI treated cells.

Ⅳ. Discussion

Farnesyl transferase inhibitors (FTIs) are a novel class of antitumor drugs that block the oncogenic activity of Ras. Since the FTIs lack significant cell toxicity, it raises a significant conundrum how the FTIs cause selective tumor regression. It was reported that the FTIs selectively inhibit growth of transformed cells. Mutated K-ras is found in many human cancers, particularly in >90% of pancreatic cancers and >40% of lung cancers.[28] Inhibition of farnesylation is expected to be an appropriate target of chemotherapy for cancers bearing ras mutations, especially for pancreatic cancers which show poor response to noted anticancer drugs.[25] Numerous investigators have pursued pharmacological means by which to inhibit Ras in the face of oncogenic mutations, notably by inhibiting the post-translational modification of Ras. However, it is unknown how the FTIs affect human cancers in detail. The biological roles of the inhibitors are still unknown, particularly the growth inhibitory effects in the presence or absence of Ras mutations.[29] Also, it is generalized that many of the effector molecules, such as AKT or ERK, are involved in the tumor regression by FTIs.

In this present study, the inhibitory effect of FTIs in RIE-1 cells with or without Ras transformation was investigated, and two Ras types, H- or K-Ras, were compared to the control cells. Furthermore, the mechanism accounting for the cell cylce effects of the FTIs was invesigated.

The RIE-1 cells, a diploid and nontransformed cell line derived from rat small intestine,[30] was stably transfected with control vector construct Neo4F and with constructs encoding activated H- or K-Ras4B, and the Western blot analysis confirmed that the transfection was done succesfully (Figure 2). Initial study was designed to demonstrate the growth inhibitory effect of FTIs, LB42908 and L744,832. (Figure 1) L744,832 selectively inhibited proliferation of activated H-ras transfected RIE-1 cells as measured by a dose dependent reduction in the level of optical density measured by MTT assay (Figure 3), whereas all three cell lines showed dose dependent suppression of the cell growth when treated with LB42908. However, while 50% growth inhibition in neo-RIE-1 cells and K-Ras RIE-1 cells was observed after 48 h with 10 μM of LB42908, less than 40% cells were growth inhibited by concentrations of LB42908 as low as 0.5 μM as depicted in Figure 4. Overall, both FTIs preferentially inhibited the proliferation of H-Ras transformed cell, with K-Ras transformed or normal cells either being resistant to these agents or being inhibited at higher doses. This relative resistance of K-Ras -transformed cell lines to FTIs is due to that K-Ras and N-Ras but not H-Ras, can be posttranslationally modified by GGTase I,[31,32] a related enzyme which transfers a 20-carbon prenyl group from geranylgeranyl pyrophosphate to cysteine residues in protein. Thus, LB42908 and L744,832 selectively and efficiently cause cell growth suppression of H-Ras transformed cells lines, while K-Ras RIE-1 cells and normals cells shows resistance to such drugs.

Previous investigations have addressed the effects of FTI on cell

cycle regulation in various cells. Inhibition of G1/S or G2/M transition appears to be the predominant cell cycle effect induced by FTI. Whereas FTI induces apoptosis in a p53-independent manner, FTI-induced G1 cell cycle arrest appears to be dependent on both p53 or $p21^{WAF1/CIP1}$. Unfortunately, no such effects of FTI on cell cycle regulation have been reported, and these variable results might be due to the use of different cell lines with multiple genetic background and usage of different types of FTIs. In pancreatic cancer cell lines, L744,832 induced the accumulation of the cells with a tetraploid(4N) DNA content and high levels of cyclin B1/cdc2 kinase activty, implying cell cycle arrest downstream form the DNA damage-inducible G2/M cell cycle checkpoint.[33] However, because the most pancreatic cancer cells are assoicated with multiple genetic defects, such as K-ras, p53, p16, DPC4, etc., it is not easy to explain the exact mechanims of FTI on cell cycle.

To elucidate molecular mechanism of FTI action, it is of importance to determine the induced changes in cell cycle regulating molecules causing growth arrest. Herein, the changes in cell cycle parameter was determined by measuring DNA content with FACS analysis. As a result, FTI's ability to inhibit cell growth was accompanied by cell cycle arrest. As depicted in Figure 5, LB42908 resulted in an increase in the proportion of G1 cells at the expense of cells in S phase, whereas the proportion of the cells in G2 and mitosis remained largely unchanged in H-Ras RIE-1 while little or no apparent change was observed in K-Ras RIE-1 or RIE-1 neo cells. In other

words, the FTI selectively blocked G1/S transition of H-Ras transformed cells without affecting the cell cycle distribution of the normal cells. Same results were obtained in response to L744,832 treatment as illustrated in Figure 5. It was reported earlier that FTIs caused G2/M arrest in pancreatic cancer cell lines.[33] Thus, whether the FTI regulates components involved in G1 or G2 check points is highly cell specific. Hence, in Ras transformed RIE-1 cells, farnesylated proteins must be involved in the G1 to S phase transition of the cells cycle, or a cell cycle regulator must be affected by FTI directly or indirectly.

In response to FTI treatment, changes in cell cycle distribution which causes G1 arrest, was observed only in cells with activated ras with more sensitivity observed in H-Ras RIE-1 cells (Figure 7). Furthermore, Ras has been implicated in the positive regulation of the cyclin D1 promotor. Thus, inhibiting Ras might effect cyclin D1 expression in a negative way. Thus, change in cyclin D1, only the earliest of the cell cycle regulators to be affected by Ras, expression was investigated. In response to LB42908 (10 μM), FTI suppressed cyclin D1 expression in H-Ras RIE-1 or K-Ras RIE-1 cells whereas the control cells showed increased expression in cyclin D1 expression as shown in Figure 8. Similar effect was observed when treated with L744,832. Since FTI also induces a G1 block in Ras transformed RIE-1 cells, the results suggest that FTIs induces G1 block through a suppression of cyclin D1 expression. Then the expression of cyclin A, a cell cycle regulator which appears in late G1 or early S to associate

with CDK2 and degraded at metaphase, was observed to determine whether FTIs inhibit S phase progression. According to Figure 8, the level of cyclin A expression was completely reduced in H-Ras RIE-1 cells, and slight decrease was observed in K-Ras RIE-1 cells whereas the RIE-1 neo cells showed no change in cyclin A expression. It suggests that the FTIs block the cyclin A accumulation and S phase progression of the cells. Also, FTI selectively and effectively blocked cyclin A activity in H-Ras transfected cells.(Figure 8) This specificity correlates with the specific blockage of G1/S transition of the H-Ras RIE-1 cells treated with FTIs. Thus, H-Ras transformed cells showing higher sensitivity toward FTIs might be due to the action of cyclin A or a regulator upstream of cyclin A. It is predicted that the blockage effect of FTI in G1/S transition is due to cyclin A depletion at the early S phase. Furthermore, whereas the cyclin A decrement is an direct effect of FTI, the change observed in the level MAPK and cyclin D1 activity is believed to be a secondary effect. However, the exact point at which the FTI action on cyclin A comes into play still needs to be elucidated.

Another protein that is required to pass the G1 restriction checkpoint and enter the S phase in different cell types is the ERK 1/2.[26,38] The ERK cascade is likely to regulate some mid late changes in gene expression that are rate limiting events for S phase entry during the G1 progression of the cell cycle.[34,35] Also, it is one of the major mitogenic signalling pathways downstream of Ras involves the serial activation of protein kinases in the Mitogen Activated Protein Kinase (MAPK) pathway.[36-38] Also, p42/p44 ERK was found to be a positive

regulator of cyclin D1 expression as it was reported by Lavoie and colleagues.[35] The mechanism by which FTIs confer their antitumorigenic effect was initially presumed to be on the basis of Ras pathway inhibition. For such reason, the effect of FTIs on the activation of MAPK was evaluated. As a result, while H-Ras RIE-1 cells, which demonstrated the greatest sensitivity to growth inhibition by both the L744,832 and LB42908, demonstrated a marked reduction in MAPK activity (Figure 10), the K-Ras RIE-1 and control cells showed only a modest inhibition of MAPK activity, which also correlates with the changes in cyclin A expression. In addition, the farnesylation of the K-Ras RIE-1 cells or the control cells is blocked by different concentration of FTIs that block H-Ras. From an earlier report by Reiss et al,[39] the isolated CAAX farnesyltransferase has a higher affinity for the tetrapeptides CVIM and CVVM, which correspond to the COOH termini of K-RasB and N-Ras, than it does for CVLS, which is the terminus of H-Ras. Thus, it might be more difficult for some FTIs to inhibit farnesylation of K-Ras or H-Ras in intact cells. However, mechanism of the little effect exerted by FTIs in normal cells is unknown and needs to be elucidated.

Finally, another Ras effector molecule PI 3-Kinase activation has been found to be required for cyclin D1 protein expression and S-Phase entry in fibroblasts.[14] In addtion, it was found to be more predominant in fibroblast cells to take PI 3-Kinase pathway in inducing increment of the cyclin D1 expression rather than taking the MAPK pathway.[40] However, as illustrated in Figure 11, all three cell lines were not affected

by the FTIs at the same dose (10 μM) that inhibited MAPK activity. Thus, it seems to be that the cell cycle arrest and down regulation of the cyclin D1 expression by FTI is not associated with PI 3-Kinase activity. Unlike those pancreatic cancer cells which undergoes apoptosis following FTI treatment by blocking AKT survival pathway, FTIs did not affect PI3 Kinase activity and did not induce apoptosis in RIE-1 cells which correlates with an earlier observation.

V. Conclusion

In conclusion, the FTIs, LB42908 and L744,832, dose dependently inhibited cell growth of H-Ras and K-Ras transfected cell lines as a result the cell cycle arrest at G1/S transition following decrement in cyclin D1 and cyclin A expression while no change was observed in normal cells treated with FTIs. In addition, the activity of ERK, a Ras effector molecule, decreased after FTI treatment in H-Ras and K-Ras RIE-1 cells while another Ras effector molecule AKT was unaffected by FTI treatment. Especially, higher sensitivity of H-Ras RIE-1 cells to FTIs correlated with dramatic change observed in MAPK and cyclin A activity whereas minimal change was shown in K-Ras RIE-1 cells and control cells. Thus, FTI is a potent antitumor agent that specifically block Ras transformed cells over normal cells. Finally, the H-Ras transformed cells are more sensitive to such drug than K-Ras transformed cells and this sensitivity is predicted to be due to FTI's mechanism through regulation of cyclin A and MAPK activity.

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국문요약

Farnesyltransferase Inhibitor의 세포주기억제를 통한 항암작용 기전에 관한 연구

Farnesyl transferase Inhibitors(FTIs)는 종양성 Ras 단백질 기능을 차단함으로서 암세포의 성장을 억제시키는 항암제로 Ras 가 활성화 되기위해서 필요한 farnesylation이라는 posttranslational modification에 관여하는 효소인 farnesyltransferase를 차단시키는 약제로 개발되었다. 그러나, 최근들어서는 FTI의 항암효과가 단지 Ras 단백질에 대한 작용에만 의거하지 않을 것이라는 견해들이 점차 제시되고 있으며 Ras 유형에 따라 다른 기전으로 작용할것이라고 발표되고 있으나 아직까지 그 주된 기전이 무엇인지는 확실하지 않다. 이 연구에서는 FTI 의 암세포 성장의 억제 기전을 밝히고자 LG에서 개발된 LB42908과 MERCK 사에서 개발된 L744,832를 이용하여 H-Ras, K-Ras cDNA를 Rat Intestinal Epithelial cell (RIE-1)을 형질 변환시킨 각 세포주에 FTI의 영향을 비교 분석하였다. 세포성장억제 효과를 MTT assay로 확인한 결과 FTI 농도에 비례 하여 H-Ras RIE-1 세포의 성장이 다른 K-Ras RIE-1 이나 RIE-1 neo 에 비해 크게 억제되었다. FACS 분석 결과에 따르면 H-Ras RIE-1 과 K-Ras RIE-1 세포들은 10μM의 농도로 48시간 처리했을 때 G0/G1 이 증가하면서 S 주기가 크게 감소하는 반면 정상 세포는 크게 영향을 받지 않았다. 특히, H-Ras는 S 주기의 세포수가 10%이하로 감소하여 K-Ras RIE-1 세포의 2배 이상의 감소를 보였다. Western blot을 수행하여 G1/S에 관여하는 단백질인 cyclin D1 과 cyclin A, 그리고 Ras의 downstream molecule인 MAPK와 AKT 의 변화를 관찰하였다. Cyclin D1은 K-Ras RIE-1과 H-Ras RIE-1 세포 모두에서 감소하였으나 cyclin A와 MAPK는 H-Ras RIE-1 세포에서 가장 크게 감소하였다. Cyclin A 와 MAPK의 변화정도가 MTT assay와 FACS분석 결과와 일치하는 것으로 보아 FTI가

H-Ras transformed 세포에 더욱 민감하게 작용하는 기전이 cyclin D1보다는 cyclin A와 MAPK를 통한것이라고 추측된다. 그러나, G1 세포주기에 관여하는 CDK4와 CDK6 그리고 Ras의 downstream에 위치하는 AKT의 변화는 관찰할 수 없었다.

이상의 결과는 FTI의 암세포 억제 기전이 G1/S의 세포주기 억제를 통한 것이며 이것은 cyclin D1 이나 cyclin A와 같은 세포주기 조절 단백의 억제와 MAPK 활성화 억제에 기인함을 시사한다.

핵심되는 말: Ras, FPTase Inhibitor(FTI), MAPK, 세포 주기
Cyclin D1, Cyclin A