Effects of BENC-511, a novel PI3K inhibitor, on the proliferation and apoptosis of A549 human lung adenocarcinoma cells

Huiqin Tian¹,², Yu Zhang¹, Qianyun Zhang¹, Shuixian Li¹, Yang Liu¹, Xiuzhen Han¹,*

¹ Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong, China; ² Department of Pharmacology, Shandong College of Traditional Chinese Medicine, Yantai, Shandong, China.

Summary
The small chemical compound 8-ethoxy-2-(4-fluorophenyl)-3-nitro-2H-chromene (S14161) was recently identified as an inhibitor of phosphoinositide 3-kinase (PI3K) and reported to inhibit tumor growth. However, its chiral structure and poor solubility prevent its further use. Compound 6-bromo-8-ethoxy-3-nitro-2H-chromene (BENC-511) is an analogue of S14161 produced by structural optimization. A previous study indicated that BENC-511 acted on multiple myeloma and that it had a toxicity by inhibiting the PI3K/protein kinase B (Akt) pathway. However, the effects of BENC-511 on the proliferation and apoptosis of A549 human lung adenocarcinoma cells have not been reported. The current study investigated the effects of BENC-511 on the proliferation and apoptosis of A549 cells in vitro. Results indicated that the compound BENC-511 inhibited the viability of A549 cells in a concentration- and time-dependent manner. BENC-511 suppressed proliferation and colony formation via S phase arrest. BENC-511 decreased the expression of cyclin A, proliferating cell nuclear antigen (PCNA), B-cell lymphoma-2 (Bel-2), phospho-mammalian target of rapamycin (p-mTOR), and phospho-Akt (p-Akt) and it increased the expression of p21WAF1CIP1(p21), Caspase-3 and Caspase-9. In conclusion, BENC-511 inhibited the proliferation of A549 human lung adenocarcinoma cells via S phase arrest as a result of up-regulation of p21 and reduction of Cyclin A/cyclin-dependent kinase 2 (CDK2)/PCNA complex and it induced apoptosis by reducing the mitochondrial membrane potential via the Akt/Bcl-2/Caspase-9 mitochondrial pathway of apoptosis.

Keywords: BENC-511, proliferation, apoptosis, p21, Akt

1. Introduction

Lung cancer is a common malignancy; it is a leading cause of death worldwide and a threat to health and life. The morbidity and mortality of lung cancer have significantly increased over close to 50 years. The incidence of non-small cell lung cancer (NSCLC) has been increasing in recent years, and NSCLC has become the most common type of lung cancer. NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, and it readily metastasizes, and it readily causes drug resistance compared to small cell carcinoma. NSCLC cells divide more slowly and spread relatively late. NSCLC accounts for about 80% of all lung cancer, about 75% of cases are identified in the middle-late stages, and its 5-year survival rate is very low (1).

The chemotherapy drugs that are commonly used to treat lung cancer, such as conventional cytotoxic drugs, mainly directly affect cell mitosis and the processes of DNA synthesis and repair. These drugs have a low selectivity and a high toxicity (2). Therefore, several key enzymes associated with tumor cell differentiation and proliferation have been screened as targets. Looking for inhibitors of these key enzyme is an important avenue for development of antitumor drugs today. Moreover, these small molecule inhibitors have better curative effects and cause fewer adverse reactions compared to conventional chemotherapy drugs.

Phosphatidylinositol 3-kinase (PI3K), a lipid
kinase, is a key signaling molecule and is found in all types of cells. Through the recruitment and activation of protein kinase B (Akt), a serine kinase and a main downstream target, PI3K/Akt plays an important role in the processes of cell adhesion, proliferation, survival, differentiation, apoptosis, metabolism, and other many types of cellular processes. Studies have indicated that dysregulation of PI3K/Akt signaling pathway is closely related to tumor development (3). Aberrant activation of PI3K/Akt signaling has been firmly established as a major determinant for cell growth and survival in an array of cancers. Blocking aberrant activation of the PI3K/Akt signaling pathway provides a new strategy for targeted cancer therapy (4). Thus, inhibitors of this signaling pathway would be potential anticancer agents, and particularly for cancer cells surviving and growing largely as a result of aberrant activation of PI3K/Akt signaling.

The small molecule compound S14161, 8-ethoxy-2-(4-fluorinated phenyl)-3-hydrogen nitro-2-benzopyran, has been identified as an inhibitor of PI3K and can inhibit tumor growth. However, its chiral structure and poor solubility prevent its further use (5). In order to obtain better active compounds, a novel analogue of S14161 was designed through structural optimization. This compound, 6-bromo-8-ethoxy-3-nitro-2H-chromene (BENC-511), has potent antiproliferative activity (6). Preliminary experiments indicated that BENC-511 acted on multiple myeloma and prostate cancer and that it had a toxicity by inhibiting the PI3K/Akt pathway (7). However, the effects of BENC-511 on the proliferation and apoptosis of A549 human lung adenocarcinoma cells have not been reported. The current study investigated the effects of BENC-511 on the proliferation and apoptosis of A549 cells in vitro. Results indicated that BENC-511 inhibited the proliferation of A549 cells and induced apoptosis by S cell cycle arrest and by reducing the mitochondrial membrane potential. The details of this mechanism still need to be studied further.

2. Materials and Methods

2.1. Materials and Chemicals

S14161 and BENC-511 were synthesized by Zhaopeng Liu (Figure 1) (6). Annexin V-FITC and propidium iodide (PI) were purchased from BD Pharmaningen (New Jersey, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (Missouri, USA). Fetal bovine serum (FBS) was from the American Type Cell Culture (Manassas, Virginia, USA) and was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_. The cells were fed every 2-3 days and detached using 0.05 trypsin/0.02% EDTA once they reached 70-80% confluence. Cells were plated at an appropriate density according to each experimental design.

A549 cells were seeded in 96-well plates at a density of 4,000 cells per well. Twenty-four hours later, cells were incubated with S14161 or BENC-511 at concentrations of 5, 10, and 20 μM for 24, 48, and 72 h respectively, and then cell viability was determined using an MTT assay. Cells were treated with MTT solution (final concentration, 0.5 mg/mL) for 4 h at 37°C. The supernatants were removed carefully, and then 100 μL DMSO was added to each well to dissolve the precipitate. Absorbance was measured at 570 nm on a microplate reader (Synergy HT).

2.2. Cell cultures

A549 human lung adenocarcinoma cells were purchased from the American Type Cell Culture (Manassas, Virginia, USA) and were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_.

2.3. Cell proliferation assay

A549 cells were seeded in 96-well plates at a density of 4,000 cells per well. Twenty-four hours later, cells were incubated with S14161 or BENC-511 at concentrations of 5, 10, and 20 μM for 24, 48, and 72 h respectively, and then cell viability was determined using an MTT assay. Cells were treated with MTT solution (final concentration, 0.5 mg/mL) for 4 h at 37°C. The supernatants were removed carefully, and then 100 μL DMSO was added to each well to dissolve the precipitate. Absorbance was measured at 570 nm on a microplate reader (Synergy HT).

2.4. Cell morphological analysis

A549 cells were seeded at a density of 2.5 × 10^5 cells/well in a 6-well plate and grew overnight in a humidified incubator at 37°C with 5% CO_. The next day, cells were incubated with/without BENC-511 (0.625, 1.25, 2.5, 5, and 10 μM) for 48 h. After incubation, cell morphology was examined under an inverted microscope (×200, Olympus, Japan).

2.5. Colony formation

A549 cells were seeded in 6-well plates at a density of 500 cells per well. After cells adhered overnight, cells were left alone or incubated with BENC-511 (0.25, 0.5, 1, and 2 μM) or S14161 (1 μM) at 37°C for 24 h.
After incubation, cells were cultured with medium until colonies formed. After growing for 10-14 days, cells were fixed in methanol containing 1% methylene blue, and their morphology was examined under an inverted microscope (×100).

2.6. Cell cycle analysis

The cell cycle was analyzed via flow cytometric analysis after PI staining. A549 cells (5 × 10^5) were seeded in a dish and then incubated with BENC-511 (2.5, 5, and 10 μM) for 48 h. Cells were harvested, washed, and fixed in cold 70% ethanol overnight and then suspended in a 1 mL PI solution (50 μg/mL DNase-free RNase A) for 30 min. The cell cycle was analyzed using a FACScan Flow Cytometer (Becton Dickinson, USA). The percentage of cells in the G0/G1, S, and G2/M phases was determined using the software ModFit LT 3.0 (Varity Software House, Topsham, USA).

2.7. Fluorescent staining of nuclei with H33258

A549 cells (5 × 10^4) were seeded in a dish and then incubated with BENC-511 (1.25, 2.5, 5, and 10 μM) or S14161 (10 μM) for 24 h. Cells were harvested and washed twice with PBS. Cells were then fixed with 3.7% paraformaldehyde for 10 min, washed twice with PBS, and incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. Cells were washed thrice and observed under a fluorescence microscope (IX-7, Olympus, Japan).

2.8. Detection of apoptosis with flow cytometry

FITC-Annexin V/PI double staining was performed as follows. A549 cells (5 × 10^5) were seeded in a dish and then incubated with BENC-511 (1.25, 2.5, 5, and 10 μM) or S14161 (10 μM) for 24 h. Cells were harvested and washed twice with PBS. Cells were then fixed with 3.7% paraformaldehyde for 10 min, washed twice with PBS, and incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. Cells were washed thrice and observed under a fluorescence microscope (IX-7, Olympus, Japan).

2.9. Determination of the mitochondrial transmembrane potential

A JC-1 probe was used to determine the effect of BENC-511 on the mitochondrial transmembrane potential (MTP). Briefly, A549 cells were cultured in 6-well plates at a density of 2.5 × 10^5 cells/well and incubated with BENC-511 (1.25, 2.5, 5, and 10 μM) or S14161 (10 μM) for 48 h. Then cells were incubated with an equal volume of JC-1 staining solution (5 μg/mL) at 37°C for 20 min in the dark and rinsed twice with ice-cold JC-1 staining buffer in accordance with the manufacturer’s instructions. MTP was monitored by determining the relative amounts of mitochondrial JC-1 monomers (green fluorescence, meaning lower MTP) and aggregates (red fluorescence, meaning higher MTP) using a fluorescence microscope.

2.10. Western blotting

A549 cells were cultured in 6-well plates at a density of 3 × 10^5 cells/well and incubated with BENC-511 (2.5-20 μM) or S14161 (20 μM) for 48 h. After treatment, cells were collected and lysed with RIPA buffer on ice for 30 min. The suspension was centrifuged at 13,000 g for 15 min at 4°C, and the supernatant was collected. The protein concentration in the total cell lysate was measured using the BCA protein assay kit with bovine serum albumin (BSA) as a standard. Other supernatants were stored at −80°C until Western blotting.

After addition of the sample loading buffer, protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF) (Millipore Corporation, Massachusetts, USA) in Tris-glycine buffer. The membranes were blocked with 5% (w/v) non-fat dry milk in 20 mM Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 2 h. The membranes were incubated with the appropriate primary antibodies at 4°C overnight and then washed three times and exposed to HRP-conjugated secondary antibodies in TBST containing 5% non-fat dry milk for 1 h at room temperature. The primary antibodies included cyclin A, PCNA, p21, Caspase-3, Caspase-9, p-Akt, and β-actin. The membranes were washed again with TBST three times. Antigen-antibody bands were detected using an enhanced chemiluminescence reagent kit (Millipore, Massachusetts, USA) and quantified using densitometry performed with the ChemiDoc XRS+image analyzer (Bio-Rad, California, USA).

2.11. Statistical analyses

Data were analyzed using one-way ANOVA and are presented as the mean ± S.E.M. A p value < 0.05 was considered statistically significant. All experiments were repeated at least three times. Statistical analysis was performed using the software SPSS/Win13.0 (SPSS, Inc., Chicago, IL).

3. Results

3.1. Effects of BENC-511 on the proliferation of A549 cells

To evaluate the effects of BENC-511 on A549 human
lung adenocarcinoma cells, cell proliferation was evaluated using an MTT assay. As shown in Figure 2A, exposure of A549 cells to BENC-511 and S14161 at the concentrations of 5, 10, and 20 μM for 24, 48, and 72 h resulted in decreased cell viability. Compared to S14161, 20 μM BENC-511 had a more potent anti-proliferative effect.

Cell morphology was examined under an inverted microscope after cells were treated with different concentrations of BENC-511 and S14161. As shown in Figure 2B, normal A549 cells had a uniform size and were epithelioid cells with adherent growth. Cells had a smooth cell wall and refracted light. The number of cells decreased gradually as the concentration of BENC-511 increased. Morphological changes in A549 cells were significant at BENC-511 concentrations of 1.25, 2.5, and 5 μM; cell density decreased significantly, cells gradually shrunk, and cells were round in shape; these aspects were concentration-dependent. Some of the cells incubated with 10 μM BENC-511 for 48 h died and floated.

To further confirm the effects of compound BENC-511 on cell proliferation, a colony formation assay was done. It also confirmed that compound BENC-511 inhibited the proliferation of A549 cells (Figure 2C). Compared to compound S14161, BENC-511 had a higher rate of inhibition.

3.2. Effects of BENC-511 on the cell cycle

The proportion of cells in the S phase increased markedly after incubation with BENC-511 (5 and 10 μM) 48 h. BENC-511 arrested the cell cycle in the S phase and prevented progression to the G2/M phase, and it inhibited cell mitosis and proliferation (Figure 3).
3.3. Effects of BENC-511 on apoptosis

To determine the effects of BENC-511 on the apoptosis of A549 cells, cell morphology was examined after Hoechst 33258 staining. As shown in Figure 4A, cell nuclear morphology changed significantly, with heterogeneous intensity and chromatin condensation after cells were incubated with BENC-511. Normal cells had round-shaped nuclei with a homogeneous fluorescence intensity. Cell apoptosis was detected using flow cytometry. Results indicated that Annexin V-positive cells increased with an increase in the concentration of BENC-511 (1.25, 2.5, 5, and 10 μM). Compared to S14161, 10 μM BENC-511 induced more marked apoptosis of A549 cells (Figure 4B).

3.4. Effect of BENC-511 on MTP

Mitochondria-dependent apoptosis is related to mitochondrial depolarization. JC-1 is a dual-emission potential-sensitive probe that can be used to measure the mitochondrial membrane potential and assess the apoptosis of A549 cells. Normally, JC-1 accumulates in the mitochondrial matrix to form a polymer that emits intense red fluorescence. However, once cells die, JC-1 can only exist in monomeric form in the cytoplasm due to a decrease in or loss of the membrane potential, resulting in green fluorescence. Therefore, color changes in A549 cells are a very direct reflection of MTP. As shown in Figure 5, results indicated that after A549 cells were incubated with different concentrations of BENC-511 for 48 h, JC-1 fluorescence changed gradually from red to green. BENC-511 induced mitochondrial depolarization. Compared to S14161, BENC-511 induced more marked apoptosis of A549 cells.
Figure 6. Effect of BENC-511 on expression of cyclin A, PCNA, and p21. A549 cells were incubated with BENC-511 (2.5, 5, 10, and 20 μM) and S14161 (20 μM) for 48 h. Cell extracts were subjected to Western blotting with specific antibodies. The expression of β-actin in cell lysates was used as an internal standard. Three experiments were performed. Data presented here are from one representative experiment. Bars represent the mean ± S.D. (*p < 0.05, **p < 0.01 compared to the control group, *p < 0.05 compared to S14161.

Figure 7. Effect of BENC-511 on expression of caspase-3, Bcl-2, p-mTOR, and P-Akt. A549 cells were incubated with BENC-511 (2.5, 5, 10, and 20 μM) for 48 h. Cell extracts were subjected to Western blotting with specific antibodies. The expression of β-actin in cell lysates was used as an internal standard. Three experiments were performed. Data presented here are from one representative experiment. Bars represent the mean ± S.D. (*p < 0.05, **p < 0.01 compared to the control group, *p < 0.05 compared to S14161.
indicated that BENC-511 induced apoptosis and mitochondrial dysfunction in vitro.

3.5. Effects of BENC-511 on the expression of cyclin A, PCNA, p21, caspase-3, Bcl-2, and P-Akt

Western blotting indicated that 2.5-20 μM BENC-511 reduced the expression of cyclin A and PCNA and up-regulated p21 expression in A549 cells (Figure 6). Caspase-3 and Caspase-9 are key members of the Caspase family and are closely related to cell apoptosis. Activated Caspase-3 can split the substrate poly ADP ribose polymerase (PARP), which activates endonuclease and results in DNA degradation, nuclear membrane rupture, and chromatin condensation. The current results indicated that Caspase-3 and Caspase-9 expression increased after cells were incubated with different concentration of BENC-511 (5, 10, and 20 μM). Moreover, BENC-511 (5, 10, and 20 μM) decreased the expression of Bcl-2, p-Akt, p-mTOR, and MAPK and upregulated the expression of Bax (Figure 7). This suggested that BENC-511 may induce the apoptosis of A549 cells by inhibiting Akt via the Bcl-2/Caspase-9/Caspase-3 mitochondrial pathway of apoptosis.

4. Discussion

The proliferation and apoptosis of tumor cells are extremely complex processes involving many factors, proteases, and signaling pathways. A previous study indicated that the compound S14161 is a PI3K inhibitor and that it acts on multiple myeloma and other tumors. However, its chiral structure and poor solubility prevent its further use. In order to obtain better active compounds, six S14161 analogues were obtained through structural optimization. BENC-511 plays a more significant role in the inhibition of PI3K/Akt. A previous study indicated that BENC-511 has a certain inhibitory effect on a wide variety of tumor cells, though its mechanism of action differed in different cell lines. The current study has examined the effects of BENC-511 on the proliferation and apoptosis of A549 human lung adenocarcinoma cells in vitro and the mechanism of that action.

In vitro experiments indicated that BENC-511 was effective in inhibiting the proliferation of A549 cells. Cell viability decreased in a concentration- and time-dependent manner after cells were incubated with different concentrations of BENC-511. These results were consistent with inhibition of colony formation. BENC-511 significantly increased the proportion of A549 cells in the S phase and arrested the cell cycle in the S phase. Further experiments indicated that BENC-511 induced the apoptosis of A549 cells. Cell morphology changed significantly and cell density decreased significantly after incubation with BENC-511. Changes in the mitochondrial membrane potential were detected, and the gradual change in JC-1 from red fluorescence to green fluorescence indicated that A549 cells were apoptotic after incubation with BENC-511. After incubation with different concentrations of BENC-511, the number of Annexin V-positive cells increased. Compared to S14161, 10 μM BENC-511 induced more marked cell apoptosis. The current study further explored the potential mechanism by which BENC-511 inhibited proliferation and induced apoptosis. Western blotting indicated that BENC-511 down-regulated the expression of cyclin A, PCNA, and p21 and up-regulated the expression of p21, Caspase-3, and Caspase-9 in A549 cells.

A tumor is a cell cycle disease, and once the cell cycle is dysregulated, the cells proliferate and divide indefinitely. Cyclins control cell cycle progression by regulating the activity of CDKs. Cyclin A is an important regulatory factor for G1/S and G2/M and is a dual control point for DNA replication and cell mitosis. Various cyclins promote the phosphorylation of pRb, Cdc6, and p107 through cyclin-CDK complexes. When Cyclin A is dissociated (not in a complex), the dephosphorylation of CDK substrates can occur, hampering these substrates from governing the processes of DNA synthesis and mitosis. PCNA is reported to be a marker of various tumors. PCNA can enhance the activity of DNA polymerase and DNA synthesis, and it plays an important role in progression from the G1 phase to the S phase.

P21 is located in the nucleus and consists of 164 amino acids. The amino terminus of p21 binds cyclins (amino acids 21-26) and CDK (amino acids 49-72), resulting in inhibition of the activity of cyclin-CDK complexes such as cyclin A-CDK2, cyclin D-CDK4/CDK6, and cyclin E-CDK2. The carboxy terminus binds PCNA, causing DNA polymerase delta to form a complex with PCNA or leading to a DNA holoenzyme that has trouble sliding along single-stranded DNA, thus affecting the replication of DNA. An important CDK inhibitor, p21 is undoubtedly a negative regulatory factor for the cell cycle. Several studies have indicated that a high level of p21 expression in tumor tissues can block normal cell cycle progression and suppress tumor development. A high level of p21 can hinder the kinase reaction, thus causing cell cycle arrest in the G1, G2, or S phase. The level of a cell cycle regulatory complex (the Cyclin A/CDK2/PCNA complex) plays a decisive role from the S phase to the G2/M phase. The current results indicated that BENC-511 inhibited the proliferation and induced the apoptosis of A549 human lung adenocarcinoma cells. BENC-511 arrested the S phase by down-regulating the expression of cyclin A and PCNA and up-regulating p21 expression. This may be related to a reduction in the level of the Cyclin A/CDK2/PCNA complex and expression of a high level of the cycle regulatory protein p21. Cell apoptosis involves multiple stages including depolarization of
the mitochondrial membrane potential, changes in permeability, the release of cytochrome c into the cytoplasm, condensed chromatin, and the formation of apoptotic bodies (16). There are two main types of pathways by which cell apoptosis occurs: mitochondria-mediated pathways of apoptosis and death receptor-mediated pathways of apoptosis. Stimuli such as ultraviolet radiation, heat stress, and DNA damage trigger mitochondrial apoptosis. Cell components can recognize this stimulus, signal the mitochondria, and increase the permeability of the mitochondrial outer membrane, releasing proteins in the mitochondria into the cytoplasm. The final executor of cell apoptosis are Caspases. Activated Caspase can cleave PARP, which activates endonuclease and results in DNA degradation, nuclear membrane rupture, chromatin condensation, and other typical characteristics of apoptosis. The activation of Caspase-9 is a key step in cell apoptosis that can initiate the Caspase cascade reaction and activate downstream caspase-3 to induce apoptosis. The current results indicated that BENC-511 decreased the mitochondrial membrane potential and the expression of Bcl-2 and it significantly increased the expression of Bax, Caspase-9, and Caspase-3 in the cytoplasm. This suggests that BENC-511 may induce the apoptosis of A549 cells by a mitochondrial pathway of apoptosis.

Studies of the relationship between lung adenocarcinoma and signaling pathways have identified a number of signaling pathways, such as Wnt/beta-catenin (17), Janus kinase/signal transducer and activator of transcription (JAK-STAT) (18) and PI3K/Akt/mTOR (19), and MAPKs (20). In recent years, several studies have reported that p21 and apoptosis are also closely related. p21 inhibits cell apoptosis. Upon DNA damage or other stressors, tumor suppressor p53 is activated, leading to transient expression of p21. This either triggers momentary G1 cell cycle arrest or leads to a chronic state of senescence or apoptosis. However, recent evidence suggests that p21 also acts as an oncogenic factor in a p53-deficient environment (21). The signaling pathways form complex signaling networks and interact with each other to influence the development of lung adenocarcinoma. The current results indicated that BENC-511 decreased the expression of P-Akt, p-mTOR, and MAPK and up-regulated p21 protein expression. This suggests that BENC-511 may induce the apoptosis of A549 cells by inhibiting Akt/mTOR via the Bcl-2/Caspase-9/Caspase-3 mitochondrial pathway of apoptosis.

In conclusion, the inhibition of A549 human lung cancer cells by BENC-511 is presumably related to additional signaling pathways and protein targets. Induction of apoptosis is very complex and involves many signaling pathways. Further study needs to be done to fully elucidate the mechanisms by which BENC-511 inhibited the proliferation and induced the apoptosis of A549 cells.

Acknowledgements
This work was supported by grants from the Key Research and Development Program of Shandong Province (No. 2016GSF201152) and the Natural Science Foundation of Shandong Province (No. ZR2017MH028) of P. R. China. The authors wish to thank Prof. Zhaopeng Liu for providing the compounds S14161 and BENC-511.

References


(Received January 13, 2019; Revised February 12, 2019; Accepted February 21, 2019)