The inhibitory mechanism of compound K on A549 lung cancer cells through EGF pathway: an in silico and in vitro approach

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In previous studies compound K (CK), an active metabolite ginsenoside from Panax ginseng, was shown to exhibit anti-cancer activity. However, the mechanism of CK through the EGFR/H-Ras pathway in cancer cells has not been reported so far. Therefore, we focused on the effect of CK as an EGFR and H-Ras inhibitor by in silico and in vitro studies using A549 cells. The biological activity prediction shows that CK exhibits chemopreventive, anticarcinogenic and anti-metastatic activity. Also, using molecular docking studies it has been shown that CK exhibits a strong binding energy with EGFR and H-Ras than Erlotinib. CK inhibits cell viability, decreases cell migration, induces apoptosis and strongly decreases gene expression of EGFR and H-Ras genes in vitro. This finding suggests that the EGFR pathway is involved in the anti-cancer activity of CK of EGF-enhanced A549 lung cancer cell line.

Keywords: Epidermal growth factor, lung cancer, molecular docking simulation, Panax ginseng.

Lung cancer is considered the most prevalent form of the disease worldwide. According to the World Health Organization, 1.8 million new cases have been identified in 2012, positioning it as the leading cause of cancer-related mortality worldwide1. Around 80–85% of all lung cancer incidences are registered as non-small-cell lung carcinoma (NSCLC)2. Overexpression or mutation of epidermal growth factor receptor (EGFR), a cell surface receptor, is related to the development and progression of human malignancies3–5. Previous studies reported that EGFR induces cell viability, differentiation and development6. The extracellular ligand-binding domain of EGFR interacts with different growth factors such as EGF and TGF-α, which subsequently causes homodimerization or heterodimerization7. Due to phosphorylation, tyrosine receptors activate several downstream signalling pathways that stimulate the activation of RAS proto-oncogenes8–9. RAS proteins play an important role in lung cancer cell growth, proliferation, apoptosis, disruption in cell cycle, tumour production and human malignancy metastasis8. Unfortunately, patients with NSCLC type are diagnosed when the disease has already reached an advanced stage; however, the aim of the treatment is controlling further growth of disease and to give a better life to the patient. Radio and chemotherapy are commonly used for cancer treatment, but the use of these therapies is associated with severe side effects and ultimately decrease in the lifespan of the patient10. Thus, the downregulation of EGFR tyrosine kinase might be an effective approach for lung cancer treatment.

Currently, there is considerable interest in the search for EGFR signalling antagonists among natural products11,12. The use of natural products in combination with well-known drugs for treatment of human diseases has been reported13,14. Panax ginseng Meyer is an important medicinal plant in the East Asian countries. Ginsenosides are the major active components of P. ginseng.15 In previous studies, ginsenosides have shown biological activity against several disease.16 Compound K (CK) is a promising intestinal bacterial metabolite of proto-panaxadiol (PPD)-type ginsenosides17. Several studies have reported CK as anticancer, antiinflammatory and antioxidant18–20. Nevertheless, the EGFR inhibition by CK through the downregulation of EGFR and H-Ras expression has not been reported so far. Therefore, in the present study, we focused on the anti-lung cancer effect of CK as an EGFR inhibitor by in silico approach followed by in vitro study using A549 lung cancer cells.

Properties such as absorption, distribution, metabolism, excretion and toxicity (ADMET) are important in the discovery of new drugs21. Thus we determined the ADMET properties for CK using methods previously described22. Also, we predict the possible biological activities of CK based on its chemical structure by PASS Online23.

From the Protein Data Bank we obtained the three-dimensional structure of the proteins EGFR (PDB ID: 1M17) and H-Ras (PDB ID: 5P21). The 3D structure of H-Ras contains magnesium (Mg), which is an important element for the protein–ligand interactions. Therefore, this element was not removed from the structure. Then, the heteroatom and water molecules were deleted from the structures using Notepad++ (http://notepad-plus-plus.org). Thereafter, the pdbqt files were made using Autodock 4.2.3 software. The Mg charge was added manually in Notepad++. The ligand structure of CK was drawn using ChemSketch program (ACD, Inc., Ontario, Canada) and converted to a 3D structure using Open Babel software24. The chemical structure of Erlotinib, a well-known EGFR inhibitor (control for this study), was obtained from PubChem (CID 176870). Finally, the ligand energy was minimized using PyRx 0.8 software.

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Table 1. Pharmacokinetic properties of compound K

<table>
<thead>
<tr>
<th>Properties</th>
<th>CK*</th>
<th>Range of 95% of known drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log BB for brain/blood</td>
<td>−2.384</td>
<td>−3.0/1.2</td>
</tr>
<tr>
<td>Q% human oral absorption in GI</td>
<td>55.08</td>
<td>&lt;25% is poor, &gt;80% is high</td>
</tr>
<tr>
<td>Aqueous solubility</td>
<td>−5.321</td>
<td>−6.5/0.5</td>
</tr>
<tr>
<td>Serum protein binding</td>
<td>0.367</td>
<td>−1.5/1.5</td>
</tr>
<tr>
<td>CYP2D6 inhibition</td>
<td>0.306</td>
<td>0 is non-inhibitor and 1 is inhibitor</td>
</tr>
<tr>
<td>Hepatotoxicity</td>
<td>0</td>
<td>0 is non-toxic and 1 is toxic</td>
</tr>
<tr>
<td>log P octanol/water</td>
<td>3.26</td>
<td>−0.2/6.5</td>
</tr>
<tr>
<td>Lipinski rule of five violations</td>
<td>2</td>
<td>Maximum 4</td>
</tr>
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</table>

Table 2. Drug-like activity of compound K

<table>
<thead>
<tr>
<th>Pa</th>
<th>Pi</th>
<th>Biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.984</td>
<td>0.001</td>
<td>Chemopreventive</td>
</tr>
<tr>
<td>0.972</td>
<td>0.001</td>
<td>Anticarcinogenic</td>
</tr>
<tr>
<td>0.868</td>
<td>0.005</td>
<td>Antineoplastic</td>
</tr>
<tr>
<td>0.777</td>
<td>0.001</td>
<td>Antimetastatic</td>
</tr>
<tr>
<td>0.721</td>
<td>0.005</td>
<td>Antineoplastic (lung cancer)</td>
</tr>
<tr>
<td>0.419</td>
<td>0.003</td>
<td>Antineoplastic (thyroid cancer)</td>
</tr>
</tbody>
</table>

For wound-healing/scratch assay, A549 cells were seeded in 12-well plate (2.5 × 10^4 cells/ml) and incubated at 5% CO2 and 37℃ humidified atmosphere. After 80–90% of confluence, the medium was replaced with a fresh one having EGF at 50 ng/ml (EGF-medium). Three hours later, each well was scratched using 0 μl plastic pipette tip and washed with PBS. The medium was replaced with control medium or EGF-medium, following our treatment schedules (2% FBS, 2% FBS + EGF, 2% FBS + 50 μM of CK, 2% FBS + EGF + 50 μM of CK)28. To quantify the scratched region, four randomly selected areas were photographed at ×10 under an optical microscope (Eclipse ME600L, Nikon Instruments, NJ, USA). The images were analysed by Image J program29.

For gene expression studies, TriZol reagent (Sigma, MO, USA) was used to extract total RNA from A549 cells and retro-transcribed into first-strand cDNAs using cDNA synthesis kit (Thermo Scientific, Lithuania, EU) according to the manufacturer’s instructions. Quantitative real-time PCR was performed as described previously30. Primer sequences for the markers are as follows: forward EGFR, 5'-CAA CCA AGCT CTC TTG AGG-3' and reverse 5'-GCT TTC GGA GAT GTT GCT TC-3'; forward RASA1, 5'-TGG GGT CAG ATC TAG GAG-3' and reverse 5'-AAC GGT ATG GCC ACC TCT T-3; forward β-actin, 5'-ATG AAG TGT GAC GTT GAC ATC C-3' and reverse 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3'. The conditions for amplification were: denaturation at 95℃ for 10 min, followed by 40 cycles at 95℃ for 10 s, 60℃ for 15 s and 72℃ for 20 s.

For fluorescent microscopy/Hoechst 33258 staining assay, A549 cells were seeded in 6-well plate and after

(ftp://pyrxsourceforge.net/downloads) and then saved as .pdbqt format using Autodock 4.2.3.

Autodock Vina 1.1.2 (ref. 24) was used to evaluate the protein–ligand interactions between the proteins EGFR and H-Ras, and the ligands CK and Erlotinib. The active sites Met769 for EGFR, and Thr35, Gly60 and Lys16 for H-Ras were demonstrated to be the most favourable binding sites for docking simulations22,25. Next, Discovery Studio 3.5 program (Accelrys Inc., CA, USA) was used for the analysis and the .pdb files and 3D image of the complex structure were prepared. Finally, the 2D structure for EGFR/Erlotinib, EGFR/CK, H-Ras/CK and H-Ras/Erlotinib interactions was generated using LigPlot+ v.1.4.5. (http://pyrxsourceforge.net/downloads).

CK was obtained from the ginseng genetic resource bank (Kyung Hee University, Korea). CK was dissolved in DMSO and stored at −20℃. RPMI-1640 medium with L-glutamine and HEPES (GenDEPOT, USA), FBS (foetal bovine serum) and P/S (penicillin G and streptomycin) (from Gibco-Brl, MD, USA) were acquired. Soluble MTT reagent [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Molecular Probes by Life Technology, OR, USA) and Recombinant Human EGF (236-EG; R&D Systems, MN, USA) were also obtained. Other materials used were commercial products of the highest grade available.

The A549 human lung carcinoma cell line was acquired from the Korean Cell Line Bank, Seoul, South Korea. RPMI-1640 medium supplemented with FBS (10%) and P/S (1%) was used to grow cells at 37℃ under 5% CO2 and 95% humidified atmosphere. MTT reagent was used to measure the cytotoxicity of CK20. Briefly, the 96-well culture plate was used to seed the cells at a density of 1 × 10^4 cells/ml. After incubation overnight, CK treatment was applied to the cells at different concentrations. After 24 h of treatment, 10 μl of MTT (5 mg/ml) was added to each well and incubated for 4 h. Then 100 μl of DMSO was added after removing the old medium and kept for 30 min to dissolve the formazan formed by viable. Absorbance was measured using multi-model plate reader (Bio-Tek Instrument, USA) at a test wavelength of 570 nm with a reference wavelength of 630 nm (ref. 27).
Table 3. Molecular docking results between ligands and proteins

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target protein</th>
<th>Compound</th>
<th>Macromolecule</th>
<th>Residue</th>
<th>Binding affinity (kcal/mol)</th>
<th>H-bond distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlotinib</td>
<td>EGFR</td>
<td>N1</td>
<td>N</td>
<td>MET769</td>
<td>-7.9</td>
<td>3.11</td>
</tr>
<tr>
<td>H-Ras</td>
<td>O4</td>
<td>HZ</td>
<td>LYS16</td>
<td>-8.3</td>
<td>3.17</td>
<td></td>
</tr>
<tr>
<td>Compound K</td>
<td>EGFR</td>
<td>O1</td>
<td>O</td>
<td>MET769</td>
<td>-8.2</td>
<td>3.20</td>
</tr>
<tr>
<td>H-Ras</td>
<td>O6</td>
<td>MG+</td>
<td>THR35</td>
<td>-9.2</td>
<td>2.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O5</td>
<td>HZ</td>
<td>LYS16</td>
<td></td>
<td>2.93</td>
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<tr>
<td></td>
<td>O6</td>
<td>MG+</td>
<td>SER17</td>
<td></td>
<td>2.93</td>
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<td>O6</td>
<td>HN</td>
<td>SER17</td>
<td></td>
<td>3.03</td>
<td></td>
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<tr>
<td></td>
<td>O8</td>
<td>HN</td>
<td>GLY13</td>
<td></td>
<td>3.01</td>
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<tr>
<td></td>
<td>O8</td>
<td>HN</td>
<td>GLY13</td>
<td></td>
<td>3.01</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Docking complex structure. The 3D structure of molecular docking results of EGFR between (a) Erlotinib and (b) compound K (CK). H-bond formation is clearly observed in the 2D structure indicated with arrows, as well as the bond distance (Å) for (c) Erlotinib and (d) CK.
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Figure 2. Docking complex structure. The 3D structure of molecular docking results of H-Ras between (a) Erlotinib and (b) CK. H-bond formation is clearly observed in the 2D structure indicated with arrows, as well as the bond distance (Å) for (c) Erlotinib and (d) CK.

Figure 3. The inhibitory activity of CK on A549 lung cancer cells after 24 h of treatment. Data shown are mean from three independent experiments analysed by ANOVA (***P < 0.001 vs control).

24 h, the cells were treated with different concentrations of CK with or without EGF stimulation for 24 h. After washing with 1 ml of 1 × PBS (twice) and fixation with 1 ml of 4% (V/V) formaldehyde (5 min at room temperature), the cells were stained during 20 min with 1 µg/ml Hoechst 33258 (1 ml each well). Stained cells were observed with a fluorescence microscope (Optinity, Korean Labtech).

Statistical significance of differences between values was evaluated by ANOVA. All experiments were repeated more than three times. Values are expressed as mean ± SD; differences are significant at P values <0.05. Analysis was done in Graph Pad 6.04 (La Jolla, CA 92037, USA).

Natural products and their derivatives, alone or in combination with other drugs, are used to increase their potential anticancer activity, which helps reduce their side effects. Based on our molecular docking studies for screening the pharmacological activities of P. ginseng saponin, we selected a promising ginsenoside such as CK, for the present study. Table 1 shows the pharmacokinetic properties of CK as blood brain barrier (BBB) levels, oral absorption in the gastrointestinal system, hepatotoxicity, aqueous solubility, serum protein binding affinity, CYP2D6 inhibition probability, logP octanol/water and the number or Lipinski rule violations. Table 2
Figure 4. Apoptosis activity of CK on A549 lung cancer cells. Nucleus stained with Hoechst 33258 dye demonstrates the morphological changes. Arrows indicate the apoptotic cell after 24 h of treatment.

Figure 5. Evaluation of anti-migratory activity of CK on A549 lung cancer cells measured by wound-healing/scratch assay. a, Percentage of cell migration area after 24 h of the scratch. b, Photographs of the cells taken at the moment of the scratch and after 24 h of treatment. Data shown are mean from three independent experiments analysed by ANOVA (***P < 0.001 vs control; ###P < 0.001 vs EGF).

shows the biological activities of CK such as a chemopreventive, anticarcinogenic, antineoplastic, antimetastatic, antineoplastic (lung cancer) and antineoplastic (thyroid cancer), predicted on the basis of probability of Pa (active) and Pi (inactive) properties using an on-line program to analyse the prediction of activity spectra for substances23. According to ADMET studies and PASS analysis, CK could be a candidate for further therapies in the field of cancer treatment according to high selectivity on the activity spectra as an antineoplastic candidate.

Using molecular docking simulation, we analysed the mechanism by which CK might be targeting the EGF-receptor and H-Ras oncoprotein. Our results show that the energy binding used by CK to target EGFR is lower
than Erlotinib (Table 3). Also, CK binds with EGFR through the active site, Met769 (Figure 1). In addition, we analysed the potential of CK to bind with H-Ras, a surface membrane protein, which can be activated after the phosphorylation of EGFR. Similar to our result with EGFR, CK exhibits lower binding energy than Erlotinib (Table 3). CK binds with the active sites Lys16 and Thr35 as well as the active residues Ser17 and Gly13 (Figure 2). According to our findings, we suggest a possible mechanism in which CK not only binds with the surface receptor EGFR, but can also target the surface membrane H-Ras oncoprotein, preventing the activation of this protein by other signalling pathways.

For drug development, the therapeutic effects of new compounds to induce apoptosis in cancer cells can be evaluated by staining the nucleus and observing the morphological change using fluorescent microscopy. Therefore, we examined the apoptotic activity of CK in A549 lung cancer cells. In order to determine the cytotoxicity of CK, MTT assay was used. The treatment with CK significantly reduced viability of A549 lung cancer cells in a dose-dependent manner (Figure 3). Following this, evaluation of morphological changes in the nucleus by Hoechst 33258 staining was carried out. We found that the number of apoptotic cells after CK treatment had increased by inducing changes in the nucleus such as chromatin condensation (Figure 4). Taken together, we suggest that CK decreases cell viability by induction of apoptosis in a concentration up to 50 μM.

Next, the potential antimigratory activity of CK on A549 cells was determined. A previous study reported that ouabain, a cardioactive glycoside, impedes invasion by decreasing the basal and enhanced migration in human lung cancer A549 cells. Interestingly, it is observed that CK strongly inhibits the motility of lung cancer cells with or without EGF-stimulation (Figure 5), suggesting that this might be an antimigratory activity of CK.

Earlier, it has been reported that upregulation of EGFR plays a critical role in the pathogenesis of lung cancer by stimulating the expression levels of different transcription factors, including H-Ras gene. Therefore, to validate the mechanisms of action of CK involved in the inhibition of cell growth, migration and apoptosis effect in A549 cells, we further evaluated the inhibitory effects of CK on mRNA levels of related genes such as EGFR and H-Ras in A549 after stimulation with EGF. Our finding shows that CK suppresses the EGF-induced mRNA expression levels of EGFR and H-Ras genes in A549 cells after 24 h of treatment (Figure 6).

This study suggests that CK exhibits a strong bond with EGFR and H-Ras proteins. Further, treatment of CK can affect cell growth as well as induce apoptosis via the suppression of EGF-stimulated EGFR and H-Ras mRNA expression levels in human lung cancer A549 cells in a dose-dependence manner. This provides a basis for future evaluation of CK and other ginsenosides for the development of new therapeutic agents as anti-lung cancer. However, further in vivo study is required for proper understanding of the mechanism of action of CK.

Conflict of interest. The authors have declared that there is no conflict of interest.


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Microbial water desalination and bio-electricity generation – role of biomass carbon

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Microbial desalination cells (MDCs) are modified microbial fuel cells (MFC) that are energy-sustainable. They use organic matter in wastewater as the energy source for desalination. The electric potential gradient is caused by exoelectrogenic bacteria. A typical MDC has a middle compartment for water desalination between the anode and cathode chambers. Our study reports lab-scale desalination, for evaluating the role of carbon from biomass waste, i.e. coconut shells. Control experiments were performed in the absence of activated carbon. Different initial salt concentrations (25 and 35 g l−1) were investigated. MDC produced a maximum voltage of 460 ± 13 mV simultaneously removing about 83.3 ± 1.3% of Na+ and 57.8 ± 1.1% of Cl− in the desalination cycle. The control MDC produced a maximum of 260 ± 8 mV and 69.3 ± 2% of Na+ removal and 51 ± 1.5% Cl− removal. These results explain the role of using activated carbon for improved power production and water desalination.

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