NOVEL APPROACH TO THE HEDGEHOG SIGNALING PATHWAY: COMBINED TREATMENT OF SMO AND PTCH INHIBITORS

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ABSTRACT

Purpose: Abnormal Sonic Hedgehog signaling Pathway (Shh) activation is crucial for the development of cancer stem cells, neoplastic growth, and epithelial-mesenchymal transition processes in adulthood. Activation of the Hedgehog signaling pathway may induce the changes in cilia found in the cell membrane, initiates the Gli1 transcription factor that is translocated to the cell nucleus and finally, the target genes are transcribed. In this study, investigation of the antiproliferative, anti-invasive, and antimigrative effect of the combined use of robotnikinin (Ptch1 antagonist) and vismodegib (Smo inhibitor) on the hedgehog signaling pathway was aimed.

Material and Methods: After demonstrating the presence of the hedgehog signaling pathway in the glioblastoma cell line U87-MG, the effect of the combined use of the robotnikinin and the vismodegib on the hedgehog signaling pathway was investigated. In vitro cell proliferation, migration, and invasion analysis of the combination of antagonist and inhibitor and in silico drug-likeness analysis were performed.

Results: Two different combinations of robotnikinin and vismodegib were tested. In vitro studies show that the combined use of agents in combined treatments of Smo and Ptch1 is more effective than their individual usage.

Conclusion: Inhibition of the hedgehog signaling pathway with specific inhibitors and antagonists is considered an innovative strategy for cancer therapy.

Keywords: Glioblastoma, hedgehog signaling pathway, signaling pathway inhibitors, robotnikinin, vismodegib

INTRODUCTION

The Hedgehog (Hh) signaling pathway was first discovered in the 1980s by Wieschaus and Nüsslein-Volhard in Drosophila melanogaster, the vinegar fly, and is named after the hair-like structures resembling hedgehog spines in mutant embryos (1) and in the early 2000s, it was beginning to be elucidated invertebrates (2). Hh regulates many developmental processes during embryogenesis, including cell cycle determination, cell proliferation, the DNA mismatch repair mechanism, homeostasis, and tissue cover development (2-3-4). It has been stated that in adulthood, abnormal activation of Sonic Hedgehog (Shh) is crucial in the evolution of epithelial-mesenchymal transition (EMT), cancer stem cells, and the neoplastic growth process (5-6-7-8-9). Shh promotes a signal transduction cascade that leads to the glioma-associated transcription factors (Gli) activity by affecting the Smoothened (Smo) and Patched 1 (Ptch1) transmembrane proteins (5). In the lack of Shh signaling, Ptch1 (the 12-transmembrane receptor) acts catalytically to suppress the Smo (7-transmembrane protein) activity and it prevents the localization to the cell surface (10–11). Activation of
the Hh signaling pathway generates changes in the cell membrane of the cilia. Ptch exits the GPR161 cilia and enters through the plasma membrane via lateral transport or directly through an intracellular vesicle, causing cascading phosphorylation of Smo (12). After this movement of the primary cilia, the cytoplasmic complex including the FUSED suppressor (SUFU), is modulated (11). Binding Shh to Patch removes Ptch's inhibition on Smo. Thus, when the pathway is inactive, Gli proteins dissociate from the suppressor SUFU complex with which they form a complex and become active (13–14). Thus, the target gene expression occurs with the Gli1 transcription factor translocation, which is the end product of the Hh pathway, to the nucleus (15).

Using cell-based phenotypic data, numerous synthetic regulators of Smo were discovered, and cancer treatment by Hh signaling pathway inhibition was investigated (16). Vismodegib, which is one of the most important and widely used of these inhibitors, is the first clinically available molecule as a Hh signaling pathway inhibitor agent. In the early 2000s, in vitro and in vivo experiments of vismodegib were shown the Hh pathway inhibition activity by tumor growth inhibition initiated by Ptch mutations or Hh ligand levels increment. However, with ongoing studies, Food and Drug Administration (FDA) approved it in 2012 (17). It is used as an antineoplastic agent with the code GDC-0449 and the trade name is Erivedge. Robotnikinin is a small molecule which is targeting the Shh-linked inhibition of the Hh pathway, discovered by Stanton et al in 2009 (18). In this study with Shh-LIGHT2 cells, the activity of robotnikinin was superior to that of different molecules synthesized in the laboratory environment. Robotnikinin binds to the Shh ligand, which activates the Hh pathway by binding to Ptch1. In the absence of repression on Smo, expression of the Gli1 transcription factor cannot occur.

In this study, the effect of the combined use of the robotnikinin (Ptch1 antagonist) and the vismodegib (Smo inhibitor) on the Hh signaling pathway in the glioblastoma cell line U87-MG was investigated by in-vitro (cell migration, invasion, proliferation analysis) and in silico (drug-likeness analysis) methods.

MATERIAL AND METHODS

Cell culture and inhibitor treatment

U87-MG were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid, 1% penicillin-streptomycin, and 1% L-glutamine. U87-MG cells were maintained at 37° C, 5% CO2 in a humidified incubator. After 80% confluence, cells were treated with different concentrations of inhibitors (2-10-25-50-75 µM and 10-25-50-75-100 µM for robotnikinin and vismodegib, respectively) for 48 h. After 48 h of treatment, WST-1 (cell proliferation reagent) was added to the cell culture medium as specified in the protocol and after 2.5 h, the absorbance value was taken at 450 nm wavelength.

Quantitative real-time polymerase chain reaction (qPCR) for gene expression analysis

Expression analysis in selected gene regions was performed after the cells were treated with robotnikinin and vismodegib for 48 h. After 48 h, NucleoSpin RNA Mini Kit was used for RNA isolation. The cDNAs were synthesized by Evoscript universal cDNA synthesis kit (Roche, Basel, Swiss) after RNA isolation.

The Ptch1, Smo, and Gli1 gene regions were analyzed with these cDNA patterns used on Qiagen, Rotor Gene Q with FastStart Essential DNA Green Master (Roche). qPCR was performed according to GAPDH the reference gene (housekeeping gene).

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Primer sequences were as follows: Ptch1 F: AGT GGT GGG TGG AAG TG; Ptch1 R: AGG ATT AAA CAT AGC CTC TTC TCC; Smo F: CAA GCT GTT GCT CGT TGC TGC GTC; Smo R: ATT CTC ACA CTG GGG CAT GTA; Gli1 F: CCA CCA AGC TAA CCT CAT GTC; Gli1 R: CCC GCT TCT TGG TCA ACT T.

Wound healing

U87-MG cells were seeded in 6-well culture plates to evaluate the cell migration and after 90% confluency, a scratch by sterile 20 µL pipette tips was made through the cell monolayer. PBS was used to wash the detached cells. The different doses of robotnikinin and vismodegib were added to the culture media. The cell migration was observed for 24 h and imaged at an inverted microscope. Images were analyzed as wound closure percentage compared to the control group by LSM Software Zen 2 (Blue Edition) software.

%Wound Closure: [Wound Distance T0– Wound Distance Tx] / Wound Distance T0×100

Real-time cell invasion assay

Cell invasion was measured using the xCELLigence RTCA Dual Plate instrument with CIM-plate 16
(Roche Diagnostics GmbH, Mannheim, Germany). The lower chamber of the plate was supplemented with a 10% FBS-containing medium. 1:40 diluted Matrigel (growth factor reduced basement membrane matrix, BD Biosciences, Erembodegem, Belgium) was used to coat the upper chamber. The impedance was recorded after 15 min for 100 h. xCELLigence RTCA software (vs.1.2.1) was used for data analysis and the invasion of cells was calculated as cell index (CI) percentage compared to the control group.

**Immunofluorescent staining**
The presence of F-actin was detected by phalloidin staining (Alexa Fluor™ 647 Phalloidin, Life Tech, Waltham, USA), and proliferation markers were detected by Ki-67 staining (Abcam, Cambridge, United Kingdom). U87-MG cells were washed once with ice-cold PBS and then ice-cold 4% paraformaldehyde in PBS was used for fixing the cells. The samples were washed with PBS twice. 0.1% Triton X-100 in PBS was used for permeabilization for 5 min and cells were incubated overnight with Ki-67 primer antibody at +4°C (final concentration was 0.5 µg/ml). Then, the samples were incubated with 1:1000 AlexaFluor668 secondary antibody for 2 h at room temperature in the dark. After Ki-67 staining, samples were stained with 1:40 AlexaFluor647 Phalloidin at room temperature in the dark for 30 min. Zeiss LSM 800 confocal laser scanning microscope (Carl Zeiss, Inc.) was used for sample monitoring and LSM Software Zen 2 (Blue Edition) was used for image analysis.

**In silico drug-likeness analysis**
Molecule SMILES of vismodegib and robotnikinin, 2 molecules targeting Hedgehog signaling pathway proteins were retrieved from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). The canonical SMILES of robotnikinin and vismodegib are 
\[\text{C1}\text{CC(=O)}\text{OC}\text{(CNC(=O)C(\text{CC}(=\text{O})\text{NCC2}=\text{CC}=\text{C}(=\text{C})=\text{C}2)\text{Cl})C3=\text{CC}=\text{CC}=\text{C3}}\text{and CS(=O)(=O)}\text{C1=\text{CC}=\text{C}(=\text{C})=\text{C}1\text{C}(=\text{O})\text{NC2}=\text{CC}(=\text{C}(=\text{C})\text{=C2)Cl})C3=\text{CC}=\text{CC}=\text{N3})\text{Cl, respectively. All assays were analyzed by web tools of Swiss ADME (the Molecular Modelling Group of the Swiss Institute of Bioinformatics).}

**Statistical analysis**
IBM SPSS 25.0 (Armonk, NY) software was used for statistical analysis. p <0.05 was used to denote significant differences between the treated groups and control groups for each inhibitor independently. Statistically significant was set at p < 0.05. (mean ± stdev of three independent experiments). Non-parametric Mann-Whitney U test for evaluating the significance between 2 independent variables; a non-parametric Wilcoxon test was used to evaluate the significance between 2 dependent variables.survival analysis statistics. A p value ≤0.05 was considered significant.

**RESULTS**
**Robotnikinin and vismodegib treatments inhibited cell proliferation and decreased target gene mRNA expressions**
Gli1 gene region is directly related to cell proliferation, resulting from Hh signaling pathway activation. Hh signaling pathway inhibition affects cell growth and proliferation. After 48 h of treatment with robotnikinin and vismodegib on the U87-MG cell line, 25 µM and increased doses were found to the effective doses of robotnikinin and vismodegib. IC50 of robotnikinin was not detected in-vitro because of the cytotoxic effect of a high concentration of DMSO (> 2%), and the dose was expected to be 82 µM. Robotnikinin inhibited 25% and 42% of cell viability at 25 µM and 50 µM, respectively. IC50 of vismodegib was found 75 µM. Vismodegib inhibited 11% and 20% of cell viability at 25 µM and 50 µM, respectively.

As shown in Figure 1, when combined treatments were applied at doses where cell viability remains constant, it has been observed that the drugs increase the inhibition effects with each other and provide a higher inhibitory effect than both doses alone.

**ΔΔCt analysis** was performed based on the Cq values obtained at the end of the qPCR, based on the control gene GAPDH. Relative mRNA expression levels are shown in Figure 1.

According to the results, robotnikinin inhibited the 12-transmembrane receptor Patched so that the relative Ptch1 mRNA expression level decreased. Similarly, vismodegib inhibited the 7-transmembrane protein...
Smoothened so that the relative Smo mRNA expression level decreased. Each inhibitor also affected Gli1 that is Hh signaling pathway transcription factor, and mRNA expression level. Combination treatment doses were determined according to the first effective doses for decreasing the Gli1 mRNA expression level of each inhibitor. Effective doses of combinations doses were determined as robotnikinin 25 µM+vismodegib 50 µM (combination 1) and robotnikinin 50 µM+vismodegib 25 µM (combination 2) (Figure 1).

**Figure 1.** Cell proliferation and cell viability activity of robotnikinin and vismodegib in U87-MG cell line. A) Cell viability effect of robotnikinin. Increased cell death was observed at increasing doses. B) Cell viability effect of vismodegib. Increased cell death was observed at increasing doses. C) Cell viability effect of vismodegib and robotnikinin combination treatment. Combination therapies are more effective than high-dose use of alone-treated robotnikinin and vismodegib. D-E) Graphs showing decreases in relative mRNA expression levels as a result of qPCR analyses. Robotnikinin was found to be effective on Ptch1 expression. Vismodegib was found to be effective on Smo expression. The common effect of both inhibitors is on Gli1 expression.

**Figure 2.** A) Invasion activity of robotnikinin, vismodegib and combinations in U87-MG cells. Cell index decreased in treated cells. B) Migration activity of Robotnikinin, vismodegib and combinations in U87-MG cells. The wound closure rate of the treated cells decreased.
Figure 3. Ki-67 (the cell proliferation marker) and phalloidin (the actin filament marker) staining of robotnikinin, vismodegib, and their combined use were given. A) Combined treatment decreased the amount of proliferation marker fluorescence in U87-MG cells. B) Combined treatment decreased the amount of actin filament marker fluorescence in U87-MG cells. C) Green color indicates actin filament fluorescence in cells, red color indicates Ki-67 fluorescence and blue color indicates nuclear radiation of cells. The scale bar is 50 µm.

Figure 4. A) Radar chart of robotnikinin. All physicochemical properties are in an optimal range. B) Radar chart of vismodegib. It is estimated that this molecule is not orally bioavailable because due to its saturation range, the number of carbons in the sp3 hybridization number is 0.05, although not less than 0.25. C) BOILED-Egg plot accordingly, WLOGP and TPSA values. The red circle represents P-gp substrate -, the blue circle represents P-gp substrate +. The yellow zone demonstrates BBB permanent capacity and the white zone demonstrates gastrointestinal absorption ability.
**Combination treatment decreased the migration and invasion potential of U87-MG cells**

The wound healing assay results reveal that after 48 h, wound closure was found as 85% for the control group. On the other hand, after 48 h, wound closure was found at 80% and 71.25% at 25 µM and 50 µM doses of robotnikinin compared to the control, respectively. Wound closure was found as 61.25% and 56.25% of vismodegib at 25 µM and 50 µM doses compared to control, respectively. For combination treatment, wound closures in combination 1 and combination 2 were found as 25% and 43.75% compared to the control, respectively. Combination 1 inhibited the wound closure 31% more compared to 50 µM vismodegib. Besides, combination 2 inhibited wound closure 27% more compared to 50 µM robotnikinin. The highest inhibition of wound closure was found for 75% in combination 1 treatment.

**Table 1.** Physicochemical and bioavailability properties of robotnikinin and vismodegib

<table>
<thead>
<tr>
<th></th>
<th>Num. rotatable bonds</th>
<th>Fraction Csp³</th>
<th>TPSA</th>
<th>Log Po/w</th>
<th>Log (SILICOS-IT)</th>
<th>Log Kp (skin permeation)</th>
<th>Synthetic accessibility</th>
<th>Leadlikeness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robotnikinin</td>
<td>6</td>
<td>0.32</td>
<td>84.50Å²</td>
<td>3.37</td>
<td>-7.63</td>
<td>-6.67 cm/s</td>
<td>4.41</td>
<td>No; 1 violation: MW&gt;350</td>
</tr>
<tr>
<td>Vismodegib</td>
<td>5</td>
<td>0.05</td>
<td>84.51Å²</td>
<td>3.87</td>
<td>-8.51</td>
<td>-6.14 cm/s</td>
<td>2.68</td>
<td>No; 2 violations: MW&gt;350, XLOGP3&gt;3.5</td>
</tr>
</tbody>
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Real-time cell invasion analysis was measured according to the impedance change created by the cells on the gold electrodes by passing through the matrigel, shown in Figure 2-A. 45th-hour invasiveness of the cells decreased with increasing doses of robotnikinin, vismodegib, and combination treatments, so real-time invasion analysis was continued until the 100th hour. As shown in Figure 2-A, when the combination 1 treatment was used on the U87-MG cell line, the invasiveness was decreased at the 48th and 100th hour, 73% and 30%, respectively; furthermore when the combination 2 treatment was used on U87-MG cell line, the invasiveness was decreased at 48th and 100th hour, 74.6% and 337%, respectively. According to these data, combination 2 was found to be a more effective dose for decreasing cell invasiveness. The most effective dose for cell invasiveness is at the 48th and 72nd hours.

In addition to all these results, cell invasiveness was decreased at 50 µM robotnikinin and 25 µM vismodegib after the 50th hour, and also these decreased at 25 µM robotnikinin and 50 µM vismodegib after the 78th hour. Although there is a decrease in cell invasiveness compared to the single-use of inhibitors, the observed change is not significant (Figure 2).

**Confocal microscopy: proliferation markers and actin filaments**

Ki-67 staining was performed for the proliferation marker and rhodamine-phalloidin staining was performed for actin filament imaging on the U87-MG cell line. After staining, images were took on confocal microscopy and the image intensity analyses were performed with LSM software Zen 2 (Blue Edition). As shown in Figure 3, compared to the control group, the intensities of phalloidin for robotnikinin and vismodegib at 50 µM decreased by 44.6% and 70%, respectively. Moreover, phalloidin intensities were found as 76.6% and 67% for combination 1 and combination 2, respectively. Combination 1 was found more effective than 50 µM vismodegib by 7%. Compared to the control group, the intensities of Ki-67 for robotnikinin and vismodegib at 50 µM decreased by 41.5% and 51.4%, respectively. Furthermore, Ki-67 intensities were found as 57.2% and 55.7% for combination 1 and combination 2, respectively. The results for Ki-67 reveal that combination 1 was found more effective than 50 µM vismodegib by 6% (Figure 3).
Comparison of drug-likeness properties of hedgehog signalling pathway inhibitors

Molecular ADME and drug-likeness analysis were performed based on the molecular physicochemical properties. Bioavailability scores calculated according to molecular physicochemical properties are shown in Table 1. Molecular bioavailability scores of vismodegib (FDA-approved), and robotnikinin (improper in vivo), were found to be similar to each other (Table 1). WLOGP-TPSA results of vismodegib and robotnikinin reveal that both molecules have human gastrointestinal absorption, but do not pass through the blood-brain barrier. Log Kp values indicate that the skin permeability of the molecules and this value is expected to be low for molecules to be taken orally. When robotnikinin and vismodegib molecules are compared, it was reported that the skin permeability of robotnikinin had a lower Log Kp value (Figure 4).

DISCUSSION

In this study, the effect of the combined use of the robotnikinin (Ptch1 antagonist) and the vismodegib (Smo inhibitor) on the Hh signaling pathway in the glioblastoma cell line U87-MG was investigated by in-vitro (cell migration, invasion, proliferation analysis) and in silico (drug-likeness analysis) methods. The results reveal that the combination of vismodegib and robotnikinin is more effective. Hh signaling depends on embryogenesis and abnormal activation in adults related to carcinogenesis. In this study, the aim is an inhibition of the most important two proteins of Hh, which are Ptch and Smo. In contrast to vismodegib is the first approved drug from the FDA to inhibit the Hh pathway. Chandra et al. (22) reported that 50-µM vismodegib treatment induced apoptosis and inhibited the cell cycle. Similarly, in this study Gli1 mRNA expression, the transcription factor of Hh signaling pathway may be more effective in cancer treatment (25). Similarly, a study by Atwood et al. showed that Smo responds to PKC-ι/λ or inhibitors of Gli2 working Smo downstream and forms the basis for the Gli antagonists' clinical usage (26).

The dual combination of the Hh signaling pathway could be evaluated as more effective than the individual agents against the Hh pathway on the glioblastoma cell line. According to the drug-drug interaction information from the DrugBank web tool (24), no combination of vismodegib drug with robotnikinin or any similar Ptch inhibitor agent was found in the clinic.
CONCLUSION
In conclusion, vismodegib and robotnikinin in combination for the treatment of glioblastoma cell line is promising. Further studies to investigate the activity of the combination deeply are strongly recommended.

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Author contribution: YB: Study and experimental design, data analysis and funding. AK: experimental design, data analysis and manuscript preparation.

Conflict of interests: None

Ethical approval: This study has been approved by the ethics committee of Dokuz Eylül University, Non-invasive Research Ethical Committee (Approval date: 09.11.2020, Decision number: 2020/27-41, protocol number: 4918-GOA).

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