Usnic acid inhibits cell proliferation via downregulation of PCNA expression in gastric carcinoma AGS cells

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ABSTRACT
Usnic acid is a secondary metabolite obtained from various species of lichen. Previous studies have shown various biological activities of usnic acid, such as anti-oxidant, anti-microbial, anti-viral, anti-protozoal, anti-inflammatory, and anti-proliferative activities in different models. Its anti-proliferative activities in gastric cancer cells are still unexplored. Herein, we have investigated the effects of usnic acid on cell proliferation and viability and associated molecular alterations in human gastric carcinoma AGS cells. The treatment of usnic acid (2.5-25µM) dose-dependently reduced cell proliferation. The mRNA expression of tumor suppressor gene phosphatase tensin homolog (PTEN) in the usnic acid-treated AGS cells was increased, which may play a role in the inhibition of cell proliferation and induction of cell death. We also observed a decrease in the expression of PCNA that regulates cell proliferation by playing an important role in DNA replication. The expression of cyclin-dependent kinase inhibitor p21, which may play a role in cell cycle and proliferation inhibition was found uninfluenced with usnic acid treatment. Thus, collectively these results revealed that usnic acid inhibits the cell proliferation of AGS cells through downregulating the expression of PCNA and can be further evaluated in vivo models for its therapeutic potentials.
revealed that usnic acid inhibits cell proliferation via modulating the expression of tumor suppressor genes.

**Materials and Methods**

**Cell Culture and Reagents**
Gastring carcinoma AGS cells (ATCC, USA) were grown in Ham’s F-12 nutrient mixture medium (Gibco BRL, Grand Island, NY) supplemented with 1% antibiotic-antimycotic solution (HiMedia) and 10% Fetal Bovine Serum (Gibco Life Technology) in a humidified 37°C incubator. (+)- Usnic acid and MTT dye were procured from Sigma Aldrich, USA, and SRL, India, respectively. Procurement of anti-PCNA, anti-p21 (CIP), and anti-GAPDH was done from CellSignaling Technology, USA.

**Cell Viability Assay**
Cell viability of gastric carcinoma AGS cells was examined through MTT assay as described earlier[6]. Briefly, AGS cells (8,000/well) were seeded in a flat bottom 96 well plate and after overnight incubation treated with 0, 2.5, 5, 10, 15, and 25 µM usnic acid for 72 h. Further, cells were processed for MTT assay.

**Semi-quantitative RT-PCR Assay**
Cells (5×10^5/plate) were seeded in 100 mm plates and after 24 h incubation treated with 0, 10, 15, and 25µM usnic acid for 48 h. Once the treatment period was over, cells harvested for RNA isolation followed by cDNA synthesis, and RT-PCR as detailed earlier.[7] Briefly, Total RNA isolation was done by Trizol Methods, then 5 µg RNA was used for cDNA synthesis. The final volume of the PCR reaction mixture was 25 µl with 3 µg of cDNA, forward and reverse primers (20 pM) and all required components. PCR tube was kept in a thermal cycler (Eppendorf, India) for 25 cycles. Agarose gel (1%)with (1µg/ml) ethidium bromide separated the amplified DNA and visualized in the GelDoc system. The following primer sequence was used: for PTEN forward primer- GCG ACG GGA AGA CAA GTT CAT, reverse primer-GCG CGC YAG CCT CTG GAT TTG ACG, for GAPDH forward primer- AAG GCT GAG AAC GGG AAG CTT GTC ATC AAT, reverse primer- TTC CCG TTC AGC TCA GGG ATG ACC TTG CCC

**Immunoblotting**
Cells were seeded and treated, as described in the previous section. After 48 h incubation, cells were lysed to prepare whole cells lysate as described.[8] The protein concentration was estimated by the Bradford method. Further, the protein was resolved in 10% denaturing SDS-PAGE gel and transferred on PVDF membrane. Skimmed milk (3%) in the blocking buffer was used to block the membrane. Subsequently, the membrane was incubated in specific antibody (diluted in skimmed milk) followed by appropriate HRP-linked secondary anti-body and the bands were detected by ECL and capture on X-ray film. The band density was analyzed by scanning the film with a high-resolution scanner and Image J program (NIH, Bethesda, MD).

**Statistical Analysis**
For statistical significance, the GraphPad Prism-5 program was used. Student’s t-test was applied to detect the degree of significance between control and treated samples. p-value of < 0.05 was considered significant.

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**Figure 1:** Effect of usnic acid on cell viability of AGS cells. AGS cells were seeded and treated with 2.5, 5, 10, 15, and 25 µM of usnic acid. After 72 h of incubation, cells were processed for MTT assay as described in Materials and Methods. Results are representative of three independent experiments. The data are presented as mean of triplicate samples for each treatment. Data points are mean of s.e.m. of three experiments. **P < 0.005, *** p < 0.001. The p-value is determined by comparing each treatment with a control group.
Results

Usnic acid causes a concentration-dependent reduction in cell viability of AGS cells.
With the aim to evaluate the anti-activity of usnic acid, AGS cells were exposed to increasing concentrations of usnic acid ranging from 2.5 to 25 µM for 72 hours. Treatment with 2.5 µM usnic acid resulted in no marked decrease in cell viability; however, at (5-25)µM concentrations showed (6-65%) decrease in cell viability after 72 h [Fig. 1].

Usnic acid induces the expression of the tumor suppressor gene and inhibits the expression of cell proliferation-inducing protein
To access the effect of usnic acid on the expression of tumor suppressor gene and proliferation-inducing proteins, AGS cells were treated with (10-25 µM) of usnic acid for 48 hours and processed for semi-quantitative PCR and western blot analysis. The result of semi-quantitative PCR revealed that usnic acid induces the expression of PTEN at 48-hour time point [Fig. 2]. Similarly, the immunoblotting analysis revealed that usnic acid decreased the expression of PCNA, which has an important role in DNA replication and hence cell proliferation [Fig. 3 (A)]. However, at a lower concentration of usnic acid, the expression of p21 has no effect, but at higher concentration (25 µM), its expression was decreased [Fig. 3(B)].

Discussion
The conventional cancer therapy has shown undesirable gastrointestinal toxicity and resistance that has limited their clinical applications. Nowadays, the focus is on the use of natural products that can treat cancer with minimum side effects. Lichens are a symbiotic consortium of nutritionally specialized fungi and green alga and/or cyanobacteria [9], which produce various biologically active metabolites with a variety of effects. One such active secondary metabolite is usnic acid, which has been studied extensively as a non-genotoxic compound with potential anticancer properties[10], including apoptotic cell death [4] and cell cycle arrest.[3, 10]. However, its biological activity against gastric cancer mortality has not been reported to date. In the present study, we have explored the anti-proliferative activity of usnic acid in gastric cancer cells. Our results primarily suggested that usnic acid strongly inhibits cell proliferation and induces cell death in human gastric cancer AGS cells.

To determine the anti-proliferative activity of usnic acid, we evaluate its effects on cell viability of gastric carcinoma AGS cells. Usnic acid resulted in a dose-dependent proliferation inhibition, which was evident by the decreased viable cell population. Indeed, the inhibition of cell growth and proliferation was sustained even after one time treatment with usnic acid, which further endorses its anticancer activity. In various cases of gastric cancer, the tumor suppressor protein PTEN (phosphatase Tensin Homolog) has been detected functionally inactive and shown to be closely associated with the development, progression, and prognosis of the disease[11]. PTEN can induce apoptosis and suppress cell proliferation by antagonizing the phosphatidylinositol 3- kinase (PI3K)/Akt signaling pathway. Our results have shown that usnic acid induces the gene expression of PTEN, which might be helpful in controlling the cell proliferation and inducing cell death of AGS cells.

On the other hand, PCNA (proliferating cell nuclear antigen) a DNA clamp that is essential for DNA replication...
and has been closely associated with cell proliferation. It has an active role in cyclin-dependent kinase regulations during cell division. Its expression is associated with metastasis and malignancies.[12-15] Hence, the expression of PCNA can be regarded as a marker of proliferating cells. Our result has shown that usnic acid dose-dependently decreases the expression of PCNA in gastric carcinoma AGS cells. However, the expression of cyclin-dependent kinase inhibitor p21 (Cip) relatively ineffective with usnic acid treatments. These results indicated usnic acid targets the PCNA expression and hence cell proliferation without modulating the expression of other cell cycle inhibitors at least, p21. Thus herein, for the first time, we have shown that usnic acid inhibits cell proliferation by downregulating the expression of PCNA in gastric carcinoma AGS cells.

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