Stigmasterol exhibits potent antitumor effects in human gastric cancer cells mediated via inhibition of cell migration, cell cycle arrest, mitochondrial mediated apoptosis and inhibition of JAK/STAT signalling pathway

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Summary

Purpose: To investigate the anticancer effects of stigmasterol on the human gastric cancer cell line SNU-1, GES-1 normal cell line and to also investigate the effects on cancer cell migration, cell cycle phase distribution, apoptosis and JAK/STAT signalling pathway.

Methods: Growth inhibitory effects were evaluated by MTT assay. Apoptosis was detected by DAPI and annexin V/PI (PI) staining. Inverted phase contrast microscopy and fluorescence microscopy were used to study the effects on cell morphology. Protein expression analysis was performed by western blotting.

Results: The results showed that stigmasterol inhibited the growth of gastric cancer cells as observed by MTT and colony formation assay. The antiproliferative effects were due to induction of mitochondrial-mediated apoptosis as indicated by DAPI and annexin V/PI staining. This was further confirmed by Bax and Bcl-2 expression. Stigmasterol also inhibited cancer cell migration and induced G2/M cell cycle arrest in a dose-dependent manner. Furthermore, stigmasterol could also inhibit the JAK/STAT signalling pathway.

Conclusion: The results of this study indicate that stigmasterol could prove beneficial in the treatment of gastric cancer and therefore further in vivo studies are required to confirm its efficacy within biosystems.

Key words: apoptosis, cell cycle arrest, gastric cancer, stigmasterol

Introduction

Plants form one of the rich source of medicines and they have been employed for treating diseases since ages [1]. Several of the drugs currently used for treatment of human ailments come from plants [2]. Diseases like malaria and cancer have been treated by plant-derived compounds. Several of the plant-derived compounds such as taxanes, podophyllotoxins and others are used for the treatment of cancer [3]. Plants function as factories producing bioactive metabolites that need further exploration. Around 70% of the world population are dependent - in one way or another - on plants for their basic primary health care [4]. Among these metabolites sterols are one such group and a number of sterols have been reported to exhibit anticancer, antimicrobial and anti-inflammatory activities to name a few [5,6]. Stigmasterol is one such compound that has been shown to possess impressive pharmacological potential [7]. A number of bioactivities have been attributed to stigmasterol which include, but are not limited to, anticancer effects [8]. However, the anticancer activity of stigmasterol has not been evaluated against gastric cancer. Gastric cancer (GC) is one of the prevalent malignancies.
and more than 50% of all cancers detected in East Asian countries are GCs. Despite a decline in GC incidence, it is still reported as one of the commonly diagnosed cancers across the world [9,10]. The primary treatment of GC is surgical resection, involving also chemotherapy in non-operable patients, but the clinical outcomes are still very poor. Besides, the adverse effects of the anticancer agents used for the treatment of GC create a negative impact on the overall health of such patients [11]. In this study we showed that stigmasterol inhibits the proliferation of the SNU-1 gastric cancer cells. JAK/STAT pathway is one of the important pathways that have been shown to play important roles in the proliferation and the tumorigenesis of several types of cancer [12] and has been shown to be activated in cancer cells [13]. In this study the anticancer effects of stigmasterol were investigated on a gastric cancer cell line by examining the JAK/STAT signaling pathway.

Methods

Cell cultures, chemicals and reagents

Human gastric cancer cell line SNU-1 and normal GES-1 cell line were procured from American Type Culture Collection. Both cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mM glutamine. The cells were cultured in incubator (Thermo Scientific, Waltham, Mass, USA) at 37°C with 98% humidity and 5% CO₂. Stigmasterol (98% HPLC-pure) and all other chemicals were procured from Sigma-Aldrich Ltd (St. Louis, Missouri, USA).

CCK-8 assay

The impact of stigmasterol on the proliferation of SNU-1 cells was investigated by CCK8 assay. The cells (5×10³) were inoculated in a 96-well plate and incubated at 37°C in an atmosphere of 5% CO₂. Following this, the cells were subjected to various doses of stigmasterol (0, 7.5, 15, and 30 μM) overnight. Thereafter, of CCK8 (10 μL) was added and incubated at 37°C for 50 min. The optical density of each well content was read at 450 nm using a microplate spectrophotometer. Cell proliferation was calculated as percentage of the control.

Colony formation assay

Gastric cancer SNU-1 cells were seeded at 200 cells/well. Thereafter, the cells were incubated for 48 hrs to allow for attachment, after which they were exposed to different doses (0, 7.5, 15 and 30 μM) of stigmasterol. The cells were then cultured for 6 days, and thereafter were washed twice with phosphate buffered saline (PBS) and treated with methanol for colony fixation. Crystal violet staining of the colonies was performed for about 30 min, and counted under light microscope.

Apoptosis assay

The gastric cancer SNU-1 cells were put at 2×10⁵ cells per well in 96-well plates and subjected to treatment with varied doses of stigmasterol (0, 7.5, 15, and 30 μM), for 24-h incubation. They were then stained with DAPI, subjected to PBS washing and then fixed with formaldehyde (10%). The DAPI-stained cells were then observed and photographed under a fluorescent microscope as described previously. For estimation of the apoptotic cell percentage annexin V/PI staining was performed as described previously [14].

Cell cycle analysis

To investigate the distribution of gastric cancer SNU-1 cells in various cell cycle phases, the stigmasterol-treated (0, 7.5, 15, and 30 μM) cells were collected and subjected to PBS washing twice. This was followed by fixation of the cells in ethanol (70%) for 1 hr. The cells were then re-suspended in PI solution (50μL/mL) and RNase1 (250μg/mL) and incubated for 30 another min at 25°C. Finally, the distribution of cells in various phases of the cell cycle was estimated using flow cytometry at 10,000 cells/group.

Western blotting

Stigmasterol-treated gastric cancer SNU-1 cells were lysed in lysis buffer and the proteins were harvested. The concentration of proteins in each sample was determined by bicinchoninic acid (BCA) assay, and Western blotting was carried out as described previously [15].

Statistics

Data were expressed as mean±SD of three biological experiments. Statistical analyses were performed using Student’s t-test by GraphPad prism 7 software. Values of p<0.01 were taken as indicating statistically significant differences.

Results

Stigmasterol inhibited the proliferation of gastric cancer SNU-1 cells

The proliferation of SNU-1 gastric cancer cells after treatment with stigmasterol was examined
by CCK-8 assay (Figure 1). The results indicated that stigmasterol could inhibit the proliferation rate of these cells and the IC$_{50}$ was 15 μM. Additionally, the antiproliferative effects were found to be dose-dependent with decreasing cell proliferation when the concentration of stigmasterol was increased (Figure 2). Of note, the anticancer effects of stigmasterol were minimal on the normal GES-1 cells. The antiproliferative effects of stigmasterol were further confirmed by the results of the colony formation assay wherein it was observed that stigmasterol could inhibit the colony formation potential of the gastric cancer cells dose-dependently (Figure 3).

**Figure 2.** Effect of stigmasterol on SNU-1 and GES-1 cell viability (mean ± SD, *p<0.05). The Figure shows that stigmasterol inhibits the viability of SNU-1 cells, while the inhibition of viability of GES-1 cells was significantly lower.

**Figure 3.** Effect of stigmasterol on the colony formation of the gastric cancer cells. The experiments were repeated three times. The Figure shows that stigmasterol inhibits the colony formation capacity of SNU-1 cells in a concentration-dependent manner.

**Stigmasterol induced apoptosis in gastric cancer SNU-1 cells**

DAPI staining was performed to investigate the underlying mechanism of the anticancer effects of stigmasterol against the SNU-1 gastric cancer cells. The results of the DAPI staining revealed that stigmasterol induced apoptosis in the gastric cancer cells with obvious blebbing, cell shrinkage and nuclear morphology (Figure 4). Next, the percentage of the apoptotic cell populations was determined by carrying out annexin V/PI double-
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Staining which showed that the percentage of the apoptotic cells increased from 1.75 to 43.66% at 30 μM of stigmasterol (Figure 5). The apoptotic effects of stigmasterol were also found to be concentration-dependent. In addition, stigmasterol also enhanced the expression of Bax and decreased the expression of Bcl-2 proteins (Figure 6).

**Figure 6.** Effect of stigmasterol on the expression of Bax and Bcl-2 as determined by western blotting. The experiments were repeated three times. The Figure shows that the expression of Bax increased and of Bcl-2 decreased upon stigmasterol treatment.

Stigmasterol triggered cell cycle arrest in gastric cancer SNU-1 cells

The effects of stigmasterol were also examined on the distribution of the SNU-1 gastric cancer cells in different phases of the cell cycle. The results showed that gastric cancer cells in the G2 phase increased substantially creating accumulation of the cells in the G2/M phase, indicative of the G2/M cell cycle arrest (Figure 7).

**Figure 7.** Effect of stigmasterol dosage on the cell cycle phase distribution of the gastric cancer cells. The experiments were repeated three times. The Figure shows that the population of the G2/M cells increased upon stigmasterol treatment (arrows depict G2/M phase cells).

Stigmasterol inhibited the migration of the SNU-1 gastric cancer cells

The effects of stigmasterol was also assessed on the migration of the SNU-1 gastric cancer cells. The results revealed that stigmasterol could suppress the migration of the gastric cancer cells in a concentration-dependent manner (Figure 8).

**Figure 8.** Effect of stigmasterol on the migration of the SNU-1 gastric cancer cells. The experiments were repeated three times. The Figure shows that the migration of the gastric cancer cells was suppressed in a concentration-dependent manner upon stigmasterol treatment.

Stigmasterol inhibited the JAK/STAT pathway

It was observed that stigmasterol could inhibit the expression of STAT1, JAK1 and JAK2. Moreover,
stigmasterol could also suppress the phosphorylation of pSTAT1, pSTAT-2, pJAK1 and pJAK2. These results reveal that stigmasterol inhibits the JAK/STAT pathway in human gastric cancer cells (Figure 9).

Discussion

Natural products have a long history of being used for the treatment of several diseases and disorders [1]. In different systems of Medicine including the Chinese system of traditional Medicine, Ayurveda and Unani Medicine, different plant extracts and decoctions are used to treat a diversity of pathological conditions [2]. In most of the cases, treatments based on these traditional remedies have produced very effective outcomes. Indeed, researchers across the world have used the knowledge from these traditional systems to isolate compounds that are effective in the treatment of several pathological conditions [3]. Several compounds that are currently used as drugs were isolated from plants. For instance, podophyllotoxins, taxanes and several other drugs were isolated from plants, and have shown tremendous therapeutic potential [19]. In this study the anticancer effects of stigmasterol were examined against the gastric cancer SNU-1 cells and observed that this compound could inhibit the proliferation and colony formation potential of these cells. These results are in concordance with several other studies wherein plant-derived sterols have been reported to exhibit anticancer effects. For instance, stigmasterol has been shown to inhibit the proliferation of breast cancer cells. Apoptosis and cell cycle arrest are two important mechanisms by which anticancer agents exert their antiproliferative effects on cancer cells. While apoptosis completely eliminates the cancer cells from the body, cell cycle arrest prevents the division of such cells with deadly results [16]. In this study, we observed that stigmasterol triggered both apoptosis and G2/M cell cycle arrest in SNU-1 cancer cells. Our results are also supported by previous studies wherein sterols have been shown to induce apoptosis as well as cell cycle arrest of cancer cells [17]. From example, a plant-derived sterol γ-Sitosterol has been shown to induce both cell cycle arrest and apoptosis of cancer cells [18]. In this study apoptosis was further confirmed by increased expression of Bax and decreased expression of Bcl-2 proteins. Metastatic cancers are often difficult to treat and the drugs that can prevent this process are considered important [19]. In the present study we observed that stigmasterol could inhibit the metastatic potential of gastric cancer cells. JAK/STAT signalling pathway is highly activated in cancer cells and plays a role in the progression of tumors [12]. Interestingly, in the present study we observed that stigmasterol caused inhibition of JAK/STAT signalling pathway, indicative of the potential of stigmasterol as a possible candidate of the treatment for gastric cancer.

Conflict of interests

The authors declare no conflict of interests.
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References


