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ORIGINAL ARTICLE

Targeted antitumor activity of Ginsenoside (Rg1) in paclitaxel-resistant human nasopharyngeal cancer cells are mediated through activation of autophagic cell death, cell apoptosis, endogenous ROS production, S phase cell cycle arrest and inhibition of m-TOR/PI3K/AKT signalling pathway

Wei Li[1]*, Guangyi Li[2]*, Wensheng She[1], Xian Hu[3], Xingyu Wu[1]

[1]Department of Otolaryngology, Ezhou Central Hospital, Ezhou City, Hubei Province, P.R. China, 436000; [2]Department of Otolaryngology, Gucheng County People's Hospital, Xiangyang City, Hubei Province, P.R. China,441700; [3]Department of Cardiothoracic Surgery, Ezhou Central Hospital, Ezhou City, Hubei Province, P.R. China, 436000.

*These two authors contributed equally to this work.

Summary

Purpose: *Nasopharyngeal carcinoma is one of common and vicious cancers of head and neck. The main purpose of this study was to examine the anticancer effects of the naturally occurring compound Ginsenoside (Rg1) against paclitaxel-resistant human nasopharyngeal cancer cells along with evaluation of its effects on cell autophagy, apoptosis, ROS production, cell cycle progression and m-TOR/PI3K/AKT signalling pathway.*

Methods: *The viability of SUNE1 cancer cell line and NP460 normal cell line was checked by CCK8 counting assay. Apoptosis-related studies were examined by fluorescent microscopy using acridine orange (AO)/ethidium bromide (EB) staining as well as flow cytometry using annexin V assay. Further, transmission electron microscopy (TEM) was used to study autophagic effects induced by Ginsenoside (Rg1). Western blot assay was used to study the effects of Ginsenoside on apoptosis and on autophagy-related protein expressions including Bax, Bcl-2, LC3-ll.*

Results: *The results indicated that Ginsenoside (Rg1) reduces the viability of the nasopharyngeal carcinoma cells in a dose-dependent manner, showing IC_{50} of 15 μM in cancer cells and IC_{50} of 80 μM in normal cell lines. The AO/EB staining showed that Ginsenoside (Rg1) inhibits the viability of cancer cells via induction of apoptotic cell death which was correlated with increase in Bax and decrease in Bcl-2 levels. Electron microscopic analysis showed that Ginsenoside (Rg1) caused the development of autophagosomes in cancer cells. Similarly, Ginsenoside (Rg1) increased the expression of LC3-II protein, indicating autophagic cell death. Ginsenoside (Rg1) also induced dose-dependent S phase cell cycle arrest. Western blot analysis showed that Ginsenoside (Rg1) has the potential to block m-TOR/PI3K/AKT signalling pathway.*

Conclusions: *In conclusion, the results of this study clearly indicate that Ginsenoside (Rg1) could be developed as a potent candidate drug against nasopharyngeal cancer provided further in vivo studies as well as toxicological studies are carried out.*

Key words: *nasopharyngeal cancer, ginsenoside (Rg1), apoptosis, autophagy, cell cycle arrest*

Introduction

Conventional chemotherapy has always been associated with toxic effects killing not only cancer cells but also the normal cells of the body. This limits the use of these chemotherapeutic agents in the

Corresponding author: Xingyu Wu, BS. Department of Otolaryngology, Ezhou Central Hospital, No. 9 Wenxing Rd, Echeng District, Ezhou City, Hubei Province, P.R. China, 436000.
Tel/Fax: +86 711 322 2091, Email: WilfredBentonirw@yahoo.com
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treatment of cancer [1]. Plants can be considered as an exceptional source of anticancer agents. Several anticancer agents have been already isolated from plants and many more are likely to be discovered in the near future [2]. The main benefit of using naturally occurring compounds for the treatment of diseases such as cancer is that most of the naturally occurring compounds are not toxic and can specifically kill cancer cells [3]. *Panax ginseng* is an important medicinal plant that has been used in the management of many diseases, cancer included, in different traditional systems of medicine [4]. Many studies including both preclinical and clinical have shown the potential of *Panax ginseng* as anticancer agent [5]. It is believed that the anticancer potential of *Panax ginseng* is mainly due to the presence of a specific group of secondary metabolites known as Ginsenosides [6]. This study was designed to examine the anticancer effects of Ginsenoside on paclitaxel-resistant human nasopharyngeal cancer cells. Nasopharyngeal cancer is one among the most common types of malignant head and neck tumors in Southeast Asia and Southern China [7]. Metastasis of nasopharyngeal carcinoma at early stage makes it one of lethal cancers [8]. The 5-year survival rate under combined treatment with adjuvant cisplatin chemotherapy and radiotherapy is 50-60% [9]. The frequent relapses and distant metastasis of nasopharyngeal cancer makes it very complicated to manage with the current treatment strategies [10]. Generally, surgical removal, systemic chemotherapy or radiotherapy are employed for nasopharyngeal carcinoma, however owing to the severe adverse effects, the patients quality of life is severely impaired [11]. Improvement of prevention through early detection and identification of the therapeutic targets may prove beneficial to curb nasopharyngeal cancer related mortality [12].

The main purpose of the current study was to investigate the anticancer effects of Ginsenoside-1 against human nasopharyngeal cancer cells on autophagy, apoptosis, ROS production and cell cycle phase distribution and m-TOR/PI3K/AKT signaling pathway.

Methods

Cell viability determination

The CCK-8 assay was used for the determination of the cell viability of the nasopharyngeal cancer cells. These cell lines were obtained from European Collection of Authenticated Cell Cultures (Salisbury, UK): nasopharyngeal cancer cell line SUNE 1 and normal cells (NP460). In brief, transfected SUNE1 cells were seeded in 96-well plates and treated with varied concentrations of Ginsenoside (Rg1) at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C with 5% CO_2 and 95% O_2. Optical density (OD_{450}) was taken with the help of a microplate reader to determine the cell viability.

AO/EB and ROS estimation

The SUNE1 cells (0.6×10^6) were seeded in 6-well plates and treated with varied concentrations of Ginsenoside (Rg1) for 24 h at 37°C. Around 10 µl cell cultures were put onto glass slides and stained with a solution of AO/EB. The slides were cover-slipped and assessed by fluorescent microscopy. ROS estimation was performed as described previously [13].

Cell cycle analysis

The SUNE1 cells were treated with varied concentrations of Ginsenoside (Rg1) and cultured for 24h at 37°C. Phosphate-buffered saline (PBS) was then used to wash the cells harvested by centrifugation. The cells were then stained with propidium iodide (PI) and the cell distribution was ascertained by flow cytometry.

Transmission electron microscopy (TEM)

The SUNE1 cells were treated with 0, 7.5, 15 and 30 µM concentrations of Ginsenoside (Rg1). The cells were then subjected to fixation in a solution of 4% glutaraldehyde 0.05 M sodium cacodylate, post-fixed in 1.5% OsO4, and dehydrated in alcohol. Then, they were prepared for flat embedding in Epon 812 and observed using Zeiss CEM 902 electron microscope.

Western blot analysis

The determination of the protein expression was carried out by western blotting. The Ginsenoside (Rg1) -treated SUNE1 cells were harvested with centrifugation. The SUNE1 cells were then lysed with RIPA buffer containing a mixture of 5% of phosphate inhibitor and protease inhibitor. The cell lysates were centrifuged at 12,000 xg for 30 min at 4°C and protein concentrations were estimated using Bio-Rad protein assay (Bio-Rad Laboratories, USA). The proteins (20µL) were separated by SDS-PAGE (50-60 V for 2h) and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Next, fat-free milk was used to block the membrane at room temperature for 1h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the bands were detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalisation.

Statistics

The results are presented as values from three independent experiments with the data expressed as means±standard deviation. Differences between the groups were examined by Student's t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Ginsenoside (Rg1) on the viability of nasopharyngeal cancer cells

The CCK-8 assay was used to unveil the effects of Ginsenoside (Rg1) on the viability of the SUNE1 nasopharyngeal cancer cells. Ginsenoside (Rg1) caused a significant reduction in the viability of these cells. The effects of Ginsenoside (Rg1) on the viability of the SUNE1 cells were dose-dependent and IC_{50} of 15 μM was observed for Ginsenoside (Rg1) against the SUNE1 cells (Figure 1). However, the effects of Ginsenoside (Rg1) on the normal NP460 cells were minimal and the IC_{50} was 90 μM.

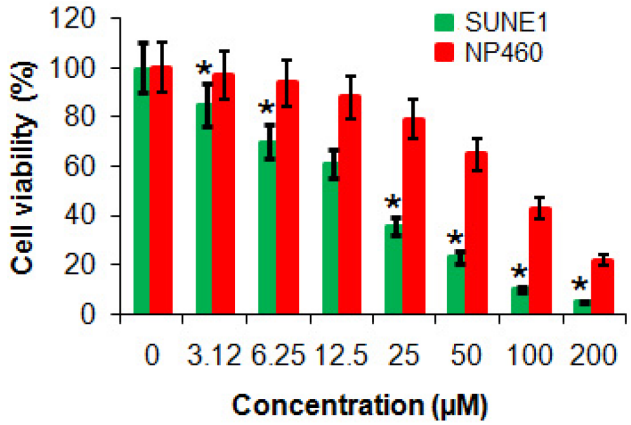

Figure 1. Cell viability assay showing the effects of ginsenoside (Rg1) on the viability of SUNE1 nasopharyngeal cancer and NP460 normal cells. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

Autophagy-inducing effects of Ginsenoside (Rg1) on nasopharyngeal cancer cells

Next, TEM of the Ginsenoside (Rg1) treated SUNE1 cells was performed. It was observed that Ginsenoside (Rg1) causes development of autophagic vesicles or autophagosomes in the SUNE1 cells which are the hallmarks of autophagy (Figure 2). Moreover, Ginsenoside (Rg1) also caused increase in the protein levels of LC3-II and decrease in the expression of p62. Nonetheless, no apparent effects were observed on the protein expression level of LC3B-I (Figure 3).

Apoptotic effects of Ginsenoside (Rg1) on nasopharyngeal cancer cells

To ascertain the underlying mechanism for the growth inhibitory property of Ginsenoside (Rg1), the SUNE1 cells were treated with different doses of Ginsenoside (Rg1) and then stained with AO/EB. The results of AO/EB assay showed that Ginsenoside (Rg1) caused nuclear fragmentation of the SUNE1 cells, characteristic of apoptosis (Figure 4). Western blot analysis showed that Ginsenoside

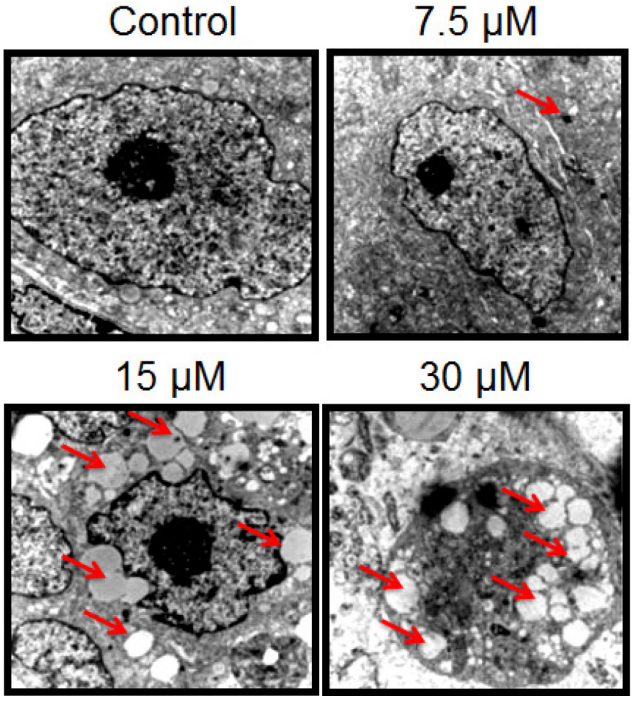

Figure 2. Electron microscopic images of SUNE1 cells treated at indicated concentrations of Ginsenoside (Rg1) showing autophagy. Arrows depict autophagosomes. The experiments were performed in triplicate.

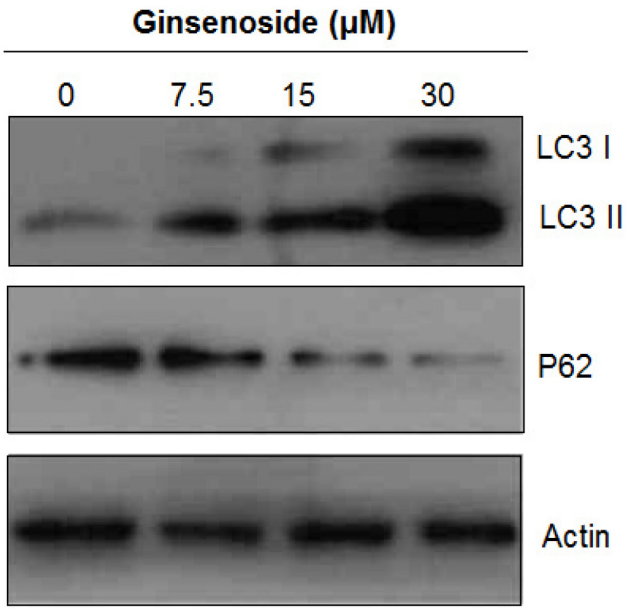

Figure 3. Effect of Ginsenoside (Rg1) on the expression of autophagy-related proteins in SUNE1 cells as depicted by western blot analysis. The expression of LC3I and LC3II increased, while of p62 decreased with increasing dose of Ginsenoside (Rg1). The experiments were performed in triplicate.

(Rg1) caused increase in the Bax and decrease in the Bcl-2 expression, confirming the apoptotic cell death in the SUNE1 cells (Figure 5). Moreover, Ginsenoside (Rg1) caused significant increase in the ROS levels of the SUNE1 cells. The ROS levels increased from 100% in the control to 220% at 30 μM concentration (Figure 6).

Ginsenoside (Rg1) caused arrest of the SUNE1 cells at the S checkpoint

The effects of Ginsenoside (Rg1) were also evaluated on the distribution of the SUNE1 cells in cell cycle phases. The results indicated that Ginsenoside (Rg1) treatment caused a significant increase in the S phase of the cell cycle, indicative of S phase arrest (Figure 7). The percentage of the S phase cells increased from 32% in the control to around 62% at 30 μM concentration of Ginsenoside (Rg1).

Ginsenoside (Rg1) deactivated the TOR/PI3K/AKT pathway in the SUNE1 cells

The effects of Ginsenoside (Rg1) were also investigated on the phosphorylation of the Raf in the SUNE1 cells (Figure 8). The results showed that the phosphorylation of mTOR, PI3K, decreased concentration-dependently upon treatment with Ginsenoside (Rg1) with no apparent effects on the total PI3K, AKT and mTOR.

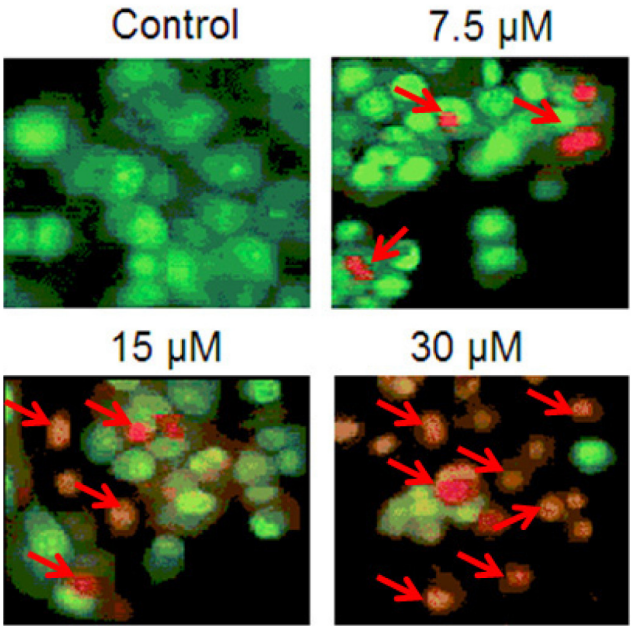

Figure 4. AO/EB staining showing that Ginsenoside (Rg1) induces apoptosis in SUNE1 cells. The results show that Ginsenoside (Rg1) caused nuclear fragmentation of the SUNE1cells, characteristic of apoptosis (red fluorescence). Arrows depict apoptotic cells. The experiments were performed in triplicate.

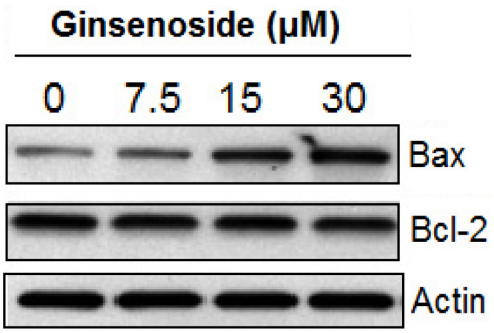

Figure 5. Effect of Ginsenoside (Rg1) on the expression of Bax and Bcl-2 proteins in SUNE1 cells as depicted by western blot analysis. The experiments were performed in triplicate. The expression of Bax increased significantly, while that of Bcl-2 decreased significantly.

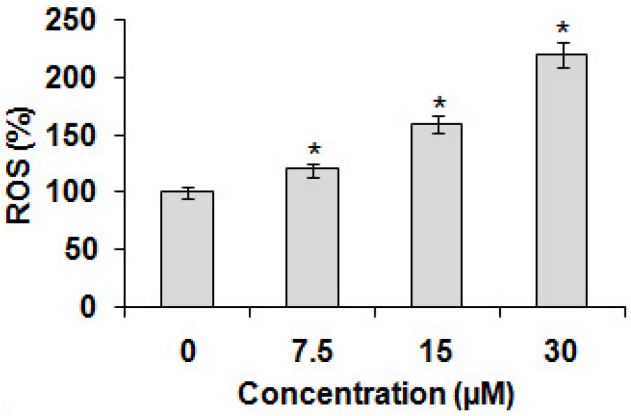

Figure 6. Effect of Ginsenoside (Rg1) on ROS production in SUNE1 cells assessed by flow cytometry. The experiments were performed in triplicate and expressed as mean±SD. Ginsenoside (Rg1) led to increase in ROS production dose-dependently (*p<0.05).

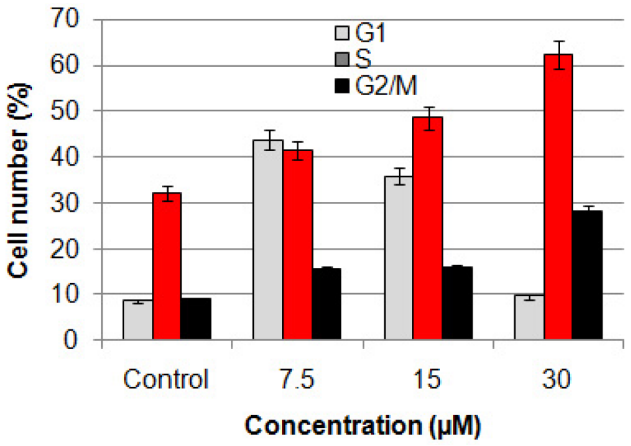

Figure 7. Effect of Ginsenoside (Rg1) at indicated concentrations on the distribution of the SUNE1 cells in different cell cycle phases. The experiments were performed in triplicate and data presented as mean ± SD. Ginsenoside (Rg1) led to significant increase in S-phase cells (S-phase cell cycle arrest).

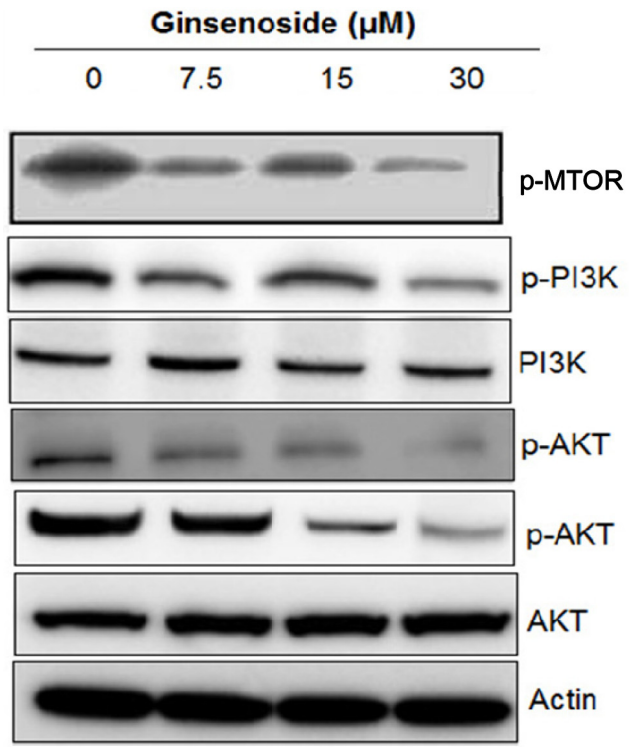

Figure 8. Effect of Ginsenoside (Rg1) on the mTOR/PI3K/AKT pathway in SUNE1 cells at indicated concentrations as depicted by western blot analysis. The experiments were performed in triplicate. The results show that the phosphorylation of mTOR, p-PI3K and p-AKT decreased concentration-dependently upon treatment with Ginenoside (Rg1) with no apparent effects on the total PI3K, AKT and mTOR.

Discussion

Nasopharyngeal carcinoma is one of the prevalent and aggressive head and neck cancers. Because of distant metastasis and significant side effects, there is persistent need to identify effective treatment options for nasopharyngeal cancers [11]. Plant-derived molecules have served as remarkable source of drugs for the treatment of deadly diseases including cancer [14]. Herein, the anticancer effects of Ginsenoside (Rg1) were examined against the human SUNE1 paclitaxel-resistant nasopharyngeal cancer cell line and normal NP640 cells. It was found that Ginsenoside (Rg1) inhibited the growth of human SUNE1 nasopharyngeal cancer cells in a dose-dependent manner. Moreover, the toxic effects of Ginsenoside (Rg1) were negligible against the human normal NP460 cells, indicating the cancer cell specific activity of Ginsenoside (Rg1). These studies are in agreement with previous studies wherein Ginsenosides have been shown to inhibit the growth of cancer cells. For example, Ginsenoside (Rg1) suppresses the growth of the nasophyrangeal MG-63 cells [15]. Moreover, Ginsenosides have been shown to trigger apoptosis and autophagy in cancer cells. For instance, Ginsenoside RH2 triggers apoptosis in colorectal cancer cells [16] and Ginsenoside F2 induces both apoptosis as well as autophagy in breast cancer stem cells [17]. Moreover, Ginsenosides have also been shown to cause both autophagy and apoptotic cell death in human melanoma cells [18]. Given these studies, the effects of Ginsenoside (Rg1) were also examined on the SUNE1 nasopharyngeal cancer cells and it was found that Ginsenoside (Rg1) triggers apoptosis and autophagy in the SUNE1 cells which was also accompanied with alteration in the marker proteins.

Cell cycle is another mechanism which enables the anticancer agents to halt the growth of cancer cells [19] and herein we found that Ginsenoside (Rg1) induces S phase cell cycle arrest. It has been reported that several signalling pathways are activated in cancer cells and mTOR/PI3K/AKT signalling pathway is also activated in cancer cells [20]. This pathway has been shown to have a role in the progression of different cancers. In this study it was found that Ginsenoside (Rg1) could block PI3K/AKT/mTOR pathway in nasopharyngeal cancer cells. Taken together, Ginsenoside (Rg1) may be considered as a possible anticancer agent in nasopharyngeal carcinoma, provided further studies are performed.

Conclusion

The present study showed that Ginsenoside (Rg1) suppresses the proliferation of human nasopharyngeal cancer cells via induction of apoptosis and autophagy. Ginsenoside (Rg1) triggers S phase cycle arrest and also blocks the mTOR/PI3K/AKT signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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