

Pine needle oil induces G2/M arrest of HepG2 cells by activating the ATM pathway

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Abstract. Over the last two decades, inducing DNA damage of cancer cells by natural medicines has become a research hotspot in the field of cancer treatment. Although various natural medicines have anticancer effects, very few studies have been conducted to explore the anti-cancer effect of pine needle oil. In the present study, the role of pine needle oil in inducing G2/M arrest in HepG2 cells was investigated. The data revealed that pine needle oil could induce DNA damage in a dose-dependent manner. In the pine needle oil-treated HepG2 cells, the protein levels of phosphorylated (p)-ataxia-telangiectasia mutated (ATM), γ -H2A histone family, member X, p-p53, p-checkpoint kinase 2 and p-cell division cycle 25C were evidently increased, indicating that pine needle oil facilitated G2/M arrest in HepG2 cells through the ATM pathway. In response to the treatment with pine needle oil, ATM was activated in HepG2 cells, which subsequently phosphorylated downstream targets and induced G2/M arrest. In summary, the data of the present study indicated that pine needle oil induces G2/M arrest in HepG2 cells by facilitating ATM activation.

Introduction

Liver cancer is one of the most common types of malignant tumor and is also one of the leading causes of cancer mortality, particularly in East Asia and Sub-Saharan Africa (1). Liver cancer has become an increasing threat to human health. The number of liver cancer mortalities is evidently increasing worldwide each year, and the 5-year survival rate is <9% (2). Additionally, the most common therapeutic options for liver cancer are surgery and chemotherapy. Therefore, it is necessary to develop novel effective drugs or preventive therapies against human hepatoma (3). Pine tree is evergreen and needle-leaved, and its various parts, including the leaf, cones, cortices and pollen, have been used as a health-promoting food and complementary therapy (4,5). Pine needle has been demonstrated to contain active components that present biological effects in several studies (6-9). As a pine needle extract, pine needle oil has demonstrated anticancer effects and has been used as an anticancer agent in traditional Chinese medicine (10).

Genomic stability is monitored throughout the cell cycle. DNA damages may result from errors during replication, by-products of metabolism, general toxic drugs or ionizing radiation (11). There are two major cell cycle checkpoints, namely G1/S and G2/M, that ensure proper cell cycle progression (12). As a critical factor to maintain genomic stability, G2/M cell cycle arrest occurs when DNA is damaged, which provides an opportunity for DNA repair and prevents cells from entering mitosis (13,14). As a serine/threonine protein kinase, ataxia-telangiectasia mutated (ATM) phosphorylates several key proteins that activate the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis (15). Several of these target proteins, including p53, checkpoint kinase 2 (CHK2), breast cancer 1, nibrin 1 and H2A histone family, member X (H2AX), are tumor suppressors (13,16-18). Therefore, dysregulation of ATM may cause defective repair of DNA damage, which results in the development of various types of cancer (19,20). The present study examined the effect of pine needle oil on the proliferation of HepG2 cells and the possible molecular mechanism underlying this effect.

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Materials and methods

Materials. Pine needle oil [dissolved in dimethyl sulfoxide (DMSO), $\leq 0.1\%$], RNase and propidium iodide (PI) solution were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti- γ -H2AX (cat no. 05636; 1:1,000) antibody was purchased from EMD Millipore Billerica, MA, USA), ATM (cat no. 2873; 1:500), p-ATM (cat no. 13050; 1:1,000), p-p53 (S15; cat no. 9286; 1:1,000), p-CDC25C (S216; cat no. 4901; 1:500) p-CHK2 (T68; cat no. 2661; 1:1,000) and CHK2 (cat no. 2662; 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). CDC25C (cat no. sc-327; 1:1,000), β -actin (cat no. sc-47778; 1:1,000), anti-H2AX (cat no. sc-54606; 1:200), p53 (cat no. sc-98; 1:500) antibodies, goat anti-rabbit (cat no. sc-2030; 1:3,000) and anti-mouse secondary (cat no. sc-2031; 1:3,000) antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Protein extraction solution kit was purchased from Beijing SBS Genetech Co., Ltd., (Beijing, China). Dulbecco's modified Eagle's medium and bovine serum albumin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The HepG2 cell line was purchased from the China Center for Type Culture Collection of Wuhan University (Wuhan, China). This cell line was originally thought to be a hepatocellular carcinoma, but is now known to be a hepatoblastoma cell line (21).

Cell culture. HepG2 cells were cultured in 1x Eagle's Minimum Essential medium (cat no. 10-009-CV; Corning Incorporated, Corning, NY, USA) supplemented with 10% FBS (cat no. 35-011-CV; Corning Incorporated). Fresh culture medium was replenished every 2~3 days. Cells were cultured at 37°C with 5% CO₂. Cell splitting was performed as follows: The cell monolayer was rinsed with 1x PBS followed by the addition of 0.05% Trypsin-EDTA solution to cover the dish bottom and then cells were incubated at 37°C for ~5 min.

Flow cytometry. The HepG2 cells were treated with pine needle oil (0.16 and 5.12 mg/ml) or DMSO (0.16 and 5.12 mg/ml) at different concentrations for 48 h at 37°C and then collected and washed with phosphate-buffered saline (PBS) twice. The cells were fixed with 70% ice-cold alcohol at 4°C for >1 h, rinsed in ice-cold PBS twice, and suspended in 3 ml PBS-containing ribonuclease (100 μ g/l; cat no. R6148-25ML, Sigma-Aldrich; Merck KGaA). Following this, HepG2 cells were incubated at 37°C for 30 min and then stained with PI (50 μ g/l) for 30 min in the dark. Subsequently, the percentages of cells at different phases of the cell cycle were analyzed by flow cytometry. The excitation wavelength utilized in the measurement was 488 nm (probe: PE-Texas Red, Biolegend, Inc. San Diego, CA, USA). Images were captured using BD FACSDiva software (v8; BD Biosciences, San Jose, CA, USA).

Western blotting. HepG2 cells treated with pine needle oil or DMSO were collected by centrifugation at 150 x g at room temperature for 10 min and resuspended with lysis buffer at a density of 2x10⁶ cells/ml followed by protein extraction using a protein extraction solution kit. Following this, the protein concentration was determined by a bicinchoninic acid assay and proteins were denatured at 95°C for 10 min. A total of

50 μ g of protein per lane was loaded into the 12% SDS-PAGE and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by blocking with TBST containing 5% BSA (cat no. BP1605-100; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Subsequently, the membrane was incubated with the primary antibodies overnight at 4°C and the PVDF membrane was washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBST) prior to incubating with the secondary antibody at room temperature for 1 h. Following incubation, the membrane was washed with TBST three times again and developed using an enhanced chemiluminescence method (Clarity Western ECL Substrate; cat no. 1705060; Bio-Rad Laboratories, Inc., Hercules, CA, USA) (22-24).

Immunofluorescence staining. HepG2 cells were treated with different doses of pine needle oil (0.16 and 5.12 mg/ml) or DMSO (vehicle control; 1:1,000) for 48 h. Cells were fixed with pre-chilled (-20°C) acetone-methanol (50:50%) for 15 min. The cells were washed with 1X PBS and then blocked with 5% BSA for 1 h at room temperature. Anti- γ H2AX antibody (cat no. 05636; 1:200; EMD Millipore) diluted in 5% BSA was added onto the slides and incubated at 4°C overnight (~15 h). The slides were then washed twice with 1x PBS (with 0.2% Triton), followed by incubation with donkey anti-mouse secondary antibody (cat no. A-21203; 1:5,000; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The cells were then mounted with anti-fade reagent containing 4',6-diamidino-2-phenylindole. A fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) was used to image the samples at a magnification of x400 (25).

Statistical analysis. Data was analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Data analyses were performed using one-way analysis of variance followed by Tukey's test. Error bars represented the mean \pm standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pine needle oil induces cell cycle arrest at the G2/M phase in HepG2 cells. In order to examine whether pine needle oil induced G2/M arrest and contributed to the inhibition of tumor growth, HepG2 cells were used as a cell model, which was treated with pine needle oil at different concentrations (0.16 and 5.12 mg/ml) in the general culture condition. DMSO was used as a vehicle control. As depicted in Fig. 1, the proportion of cells at the G2/M phase evidently increased in the pine needle oil-treated group compared with the control group. In addition, the number of HepG2 cells at the G2/M phase was significantly increased in the group treated with higher concentration of pine needle oil (5.12 mg/ml) compared with the group treated with a lower concentration of pine needle oil (0.16 mg/ml) ($P < 0.05$; Table I). By contrast, the proportion of the cell population at the G0/G1 phase was markedly decreased in the group treated with higher concentration of pine needle oil (5.12 mg/ml) compared with the group treated with a lower concentration of pine needle oil. The data in Fig. 1 and Table I indicate that pine needle oil facilitated G2/M arrest of HepG2 cells in a dose-dependent manner.

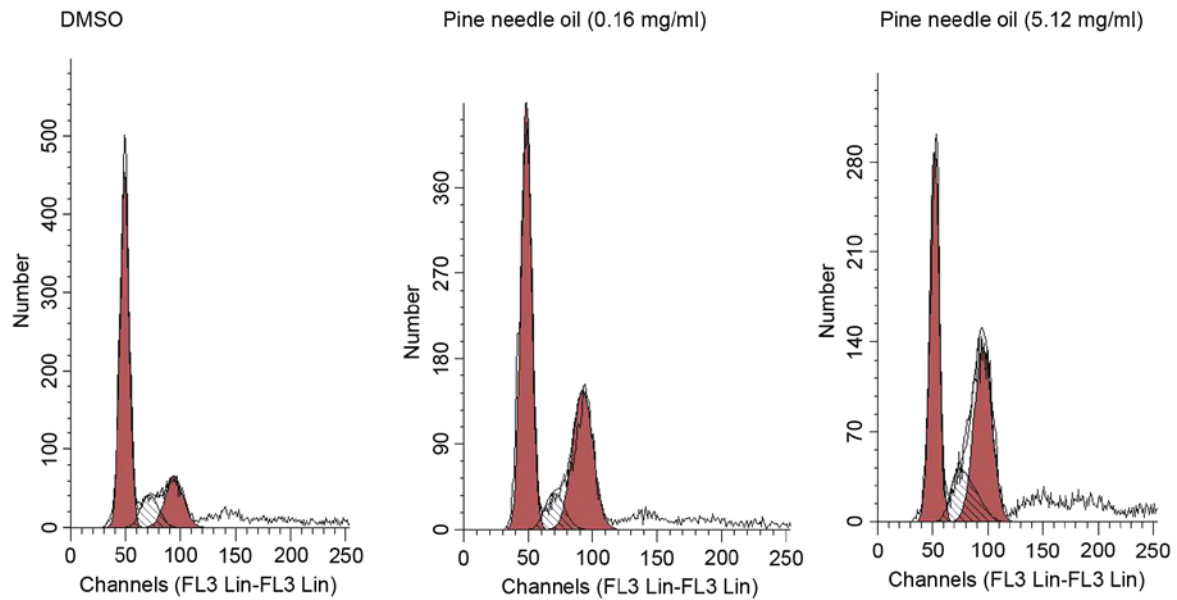


Figure 1. Treatment with pine needle oil induces cell cycle arrest at the G2/M phase in HepG2 cells. The HepG2 cells were treated with pine needle oil at different concentrations and DMSO as a vehicle control for 48 h, followed by flow cytometry analysis to determine the phase distribution in the cell cycle. The data revealed that pine needle oil induced G2/M arrest in a dose-dependent manner. DMSO, dimethyl sulfoxide.

Pine needle oil induces G2/M arrest by activating the ATM pathway. To evaluate whether cell cycle-related proteins, including p53, ATM, H2AX and CHK2, were involved in the pine needle oil-induced G2/M arrest in HepG2 cells, the expression levels of these proteins were assessed by western blotting. As demonstrated in Fig. 2, following incubation with pine needle oil at various concentrations (0.00, 0.01, 0.16, 5.12 and 40.96 mg/ml), p-ATM (S1981), p-CHK2 (T68), p-p53, p53 and γ -H2AX expression levels in HepG2 cells were markedly upregulated. Furthermore, the higher concentration of pine needle oil administered, the greater the upregulation of these proteins, which indicated that the activation of these proteins by pine needle oil occurred in a dose-dependent manner. These results demonstrate that G2/M arrest was induced by pine needle oil by activation of the ATM pathway in HepG2 cells.

Pine needle oil induces the expression of γ -H2AX in the nucleus of HepG2 cells. To further confirm the upregulation of γ -H2AX in HepG2 cells following treatment with pine needle oil, immunostaining was performed with specific antibodies against γ -H2AX. As presented in Fig. 3, without pine needle oil treatment, γ -H2AX was not detectable in the nuclei of HepG2 cells. Following pine needle oil treatment at different concentrations, γ -H2AX (red) was evidently upregulated in the nuclei in a dose-dependent manner (Fig. 3).

Discussion

Liver cancer is one of the most commonly diagnosed types of cancer worldwide and the third most common cause of cancer mortality (26,27). This disease is responsible for ~1 million mortalities per year (28). Thus far, the most recognized therapeutic strategies for liver cancer are surgery and chemotherapy. Although tremendous achievements have been made in the treatment of liver cancer over the last two decades,

the therapeutic effects of current options of treatment remain unsatisfactory and it is essential to seek more effective treatment methods to improve the prognosis of liver cancer (29). Natural medicine, particularly traditional Chinese medicine, is one of the most attractive therapeutic methods used to treat various types of cancer. Recently, efforts have been largely focused on exploring effective natural medicine for the treatment of liver cancer (30-33). Pine needle oil is one such natural medicine that may serve an important role in the treatment of liver cancer (10,34,35). It is extracted and purified from the leaf of the *Pinus*, a member of the Pinaceae family (36). Previous studies have revealed that pine needle oil has antioxidant, antimicrobial and anticancer activities (34,37). However, these studies need to be further validated, as the ability to inhibit the abnormal proliferation and induce apoptosis in human tumor cell lines could be applied to develop more effective anticancer drugs.

During the development of an organism, several types of biological processes are fundamental, including cell proliferation, division and deformation, apoptosis and necrosis (38). Regulation of the cell cycle involves processes crucial to cell survival, including the monitoring and repair of genetic damages, as well as the prevention of uncontrolled cell division (39). The molecular events that control the cell cycle are ordered and directional. Once certain abnormal activities occur at certain phases of the cell cycle, cells may enter pathological processes, such as programmed (40-42). A prevalent view is that uncontrolled cell cycle events are responsible for the initiation and development of tumors. The destruction of cell cycle regulation is a typical feature in the majority of cancer cells (43,44). Therefore, in order to control cancer, it is critical to correct the mistakes in cell cycle regulation, and the checkpoints provide the opportunity for self-healing of cells prior to mitosis. The G2/M checkpoint, also known as the DNA damage checkpoint, ensures that the cell undergoes all the necessary changes during the S and G2 phases and is

Table I. Cell cycle analysis of HepG2 cells treated with pine needle oil.

Cell cycle phase	Percentage of cells in each cell cycle phase in each treatment group		
	Dimethyl sulfoxide	Pine needle oil (0.16 mg/ml)	Pine needle oil (5.12 mg/ml)
G1	61.5±5.2	47.4±3.9 ^a	43.2±1.3 ^{b,c}
S	21.5±4.4	17.2±2.4 ^a	11.3±1.6 ^{b,c}
G2/M	17.0±1.1	35.4±3.4 ^a	45.5±2.1 ^{b,c}

Data were produced from three independent experiments and are presented as the mean ± standard error of the mean. ^aP<0.05 low dose pine needle oil group vs. DMSO group; ^bP<0.05 high dose pine needle oil group vs. DMSO group; ^cP<0.05 high dose pine needle oil group vs. low dose pine needle oil group.

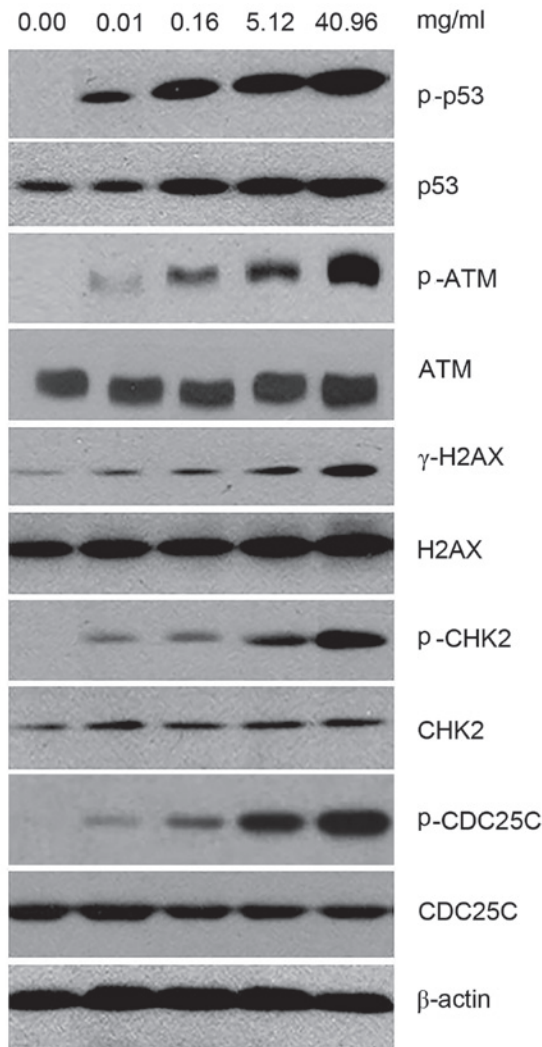


Figure 2. Key proteins involved in the ATM pathway are activated by treatment with pine needle oil. HepG2 cells treated with dimethyl sulfoxide or pine needle oil at various concentrations were lysed and analyzed by western blotting. p-ATM (S1981), p-CHK2 (T68), p-p53, p53 and γ -H2AX were evidently upregulated in HepG2 cells following treatment with pine needle oil. Additionally, β -actin was used as a loading control. p, phosphorylated; ATM, ataxia-telangiectasia mutated; H2AX, H2A histone family; CHK2, checkpoint kinase 2; CDC25C, cell division cycle 25C.

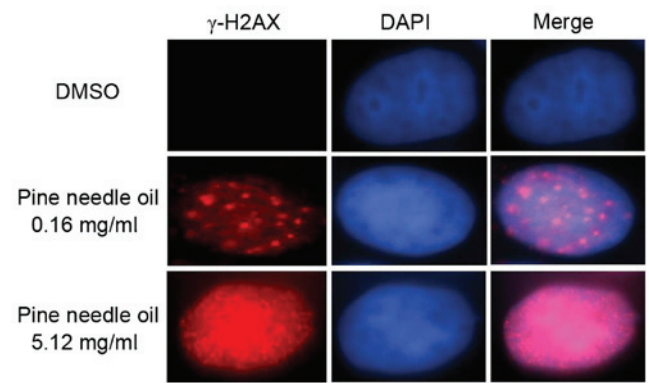


Figure 3. Treatment with pine needle oil enhances the nuclear expression of γ -H2AX. The nucleus was stained with DAPI. The treatment of pine needle oil markedly enhanced the nuclear expression of γ -H2AX (red) in HepG2 cells in a dose-dependent manner. H2AX, H2A histone family, member X; DMSO, dimethyl sulfoxide; DAPI, 4',6-diamidino-2-phenylindole. Magnification, $\times 400$.

ready for cell division (45,46). Thus, inducing G2/M arrest is an effective strategy of controlling the proliferation of cancer cells. As demonstrated in the present study, the proportion of HepG2 cells at the G2/M phase was markedly elevated when the cells were treated with pine needle oil. The results indicated that pine needle oil induced cell cycle arrest at the G2/M checkpoint in HepG2 cells and inhibited cancer cell growth by interfering with mitotic progression.

DNA damage naturally and frequently occurs due to endogenous and exogenous cellular processes (47,48). It is a major problem for life since cells must make large investments in DNA repair processes to maintain genomic integrity. The failure of self-healing of DNA damage or the accumulation of DNA damage-related factors may lead to abnormal cell growth that may result in the formation and development of diseases, such as cancer (49,50). It was reported that DNA damage may activate ATM (51,52). Furthermore, the activation of ATM subsequently facilitates the activation or phosphorylation of other key proteins that are able to detect DNA damage. Once these downstream proteins perceive the signals of DNA damage, they may trigger programmed processes to complete the repair of damaged DNA, induce apoptosis or promote cell cycle arrest (53,54). Therefore, the ATM pathway serves an important role in maintaining genomic stability and preventing tumorigenesis. Several targets of ATM, including H2AX, p53 and CHK2, are tumor suppressors (55,56). In response to DNA double-strand breaks (DSB), H2AX may be phosphorylated on serine 139 by ATM. Furthermore, the phosphorylated product of H2AX, namely γ -H2AX, is a sensitive target for DSB in cells (57,58). As a tumor suppressor, p53 has been described as 'the guardian of the genome' due to its role in conserving stability by preventing genomic mutations. Previous studies have revealed that p53 has anticancer function through various mechanisms and is involved in cell cycle arrest in HepG2 cells (59-65). As a protein that also acts as a tumor suppressor, CHK2 regulates cell division in a way to prevent cells from dividing too rapidly or in an uncontrolled manner (66,67). In 1999, genetic variations of CHK2 were revealed to be associated with inherited cancer susceptibility (68). Specifically, Thr68 of CHK2 may be phosphorylated and activated by

DNA damage-activated ATM (69). The activated CHK2 may then phosphorylate downstream targets, including cell division cycle (CDC)25C phosphatases, which may prevent the cell from entering mitosis by dephosphorylating and activating cyclin B-bound CDC2 (70). By preventing mutated or damaged DNA from being passed into daughter cells, CHK2 stops tumors from developing (71,72).

Treatment of cancer by traditional Chinese medicine has become an important therapeutic strategy for cancer patients (73-78). As a traditional Chinese medicine, pine needle oil has been used for numerous years. It was reported that pine needle oil had antioxidant and antimicrobial activities (37). In addition, scientists have revealed that pine needle oil possesses anticancer effects in various cancer cells, such as breast cancer cells (79). Previously, Wei *et al* (34) demonstrated that pine needle oil could induce the apoptosis of HepG2 cells. However, besides the data that reveals that pine needle oil could induce apoptosis and activity changes of telomerase in the HepG2 cell line (34), no further studies have been conducted. Therefore, it is crucial to conduct more in-depth investigations to comprehensively understand the mechanisms underlying the anticancer effect of pine needle oil. The present study indicated that pine needle oil induces G2/M cell cycle arrest and presents its anticancer effect by activating the ATM pathway. The data demonstrated that the expression of p-ATM (S1981), p-CHK2 (T68), p-p53, γ -H2AX and CDC25C all increased in a dose-dependent manner in the HepG2 cells that were treated with pine needle oil, implying that pine needle oil induces G2/M arrest by activating the ATM pathway. When the HepG2 cells were treated with pine needle oil, the damaged DNA activated the ATM by autophosphorylation of ATM (S1981), which subsequently facilitated the activation of CHK2, H2AX and p53 by phosphorylation. The activation of these downstream proteins further transfers the signals to other related proteins, which results in cell cycle arrest. Furthermore, it is well recognized that DNA damage may induce the phosphorylation of ATM (80,81). The confocal laser scanning microscopy data of the present study demonstrated that treatment with pine needle oil could increase p-ATM in the nucleus of HepG2 cells, indicating that DNA damage induced the activation of ATM by phosphorylation in the nucleus. Based on the data provided in the present study, a conclusion may be drawn that pine needle oil induces G2/M cell cycle arrest by activating the ATM signaling pathways, inhibits proliferation, and balances proliferation and apoptosis in HepG2 cells.

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