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Original Research

Urtica dioica agglutinin (a plant lectin) has a caspase-dependent apoptosis induction effect on the acute lymphoblastic leukemia cell line

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Abstract: Urtica dioica agglutinin (UDA) is a very small plant lectin with anti-prostatic activity. In this study, we investigated the effect of UDA on proliferation and apoptosis induction in human acute lymphoid leukemia (ALL) cell lines. The effect of UDA on Jurkat and Raji cell proliferation was examined by MTS assay. Distribution of cell cycle phases was determined by PI staining and apoptosis was examined with annexin V/PI and western blot. Results showed UDA treatment reduced cell proliferation in cells by inducing apoptosis. PI staining was associated with a higher percentage of the cell population in sub G1. Caspase-8 and caspase-9 dependent apoptosis occurred in Jurkat cells. Generally, UDA treatment resulted in cell death in ALL cell lines and induced apoptosis in the T-ALL cell line, Jurkat, through extrinsic and intrinsic pathways. These results may be considered as a guide to working on UDA as an anti-leukemic drug in the future.

Key words: Acute lymphoid leukemia; *Urtica dioica* agglutinin; Lectin**;** Anti-leukemic drug.

Introduction

Urtica dioica agglutinin (UDA) is an 8.5 KDa single-chain chitin-binding lectin, isolated from rhizomes of stinging nettle (*Urtica dioica*) (1, 2). It is the smallest plant lectin (3), with 89 amino acids including tow hevein domains (4). Lectins are a group of proteins that bind carbohydrates and play special roles in the cell agglutination, apoptosis induction and inhibition of angiogenesis, cell growth and protein synthesis. Therefore, cell regulation would be affected by these proteins (5). UDA is a super-antigen with antiviral effect and it can activate T lymphocytes. UDA also induces the production of gamma-interferon in human lymphocytes and agglutinates human erythrocytes (6). UDA is an important anti-prostatic component of *Urtica dioica* that can block the EGF receptor in prostatic tissue by binding to benign prostatic hyperplasia (BPH) cells and inhibiting proliferation (7). Anticancer effects of *Urtica dioica* root extracts have been evaluated in some studies (8-10). Methanolic extract of *Urtica dioica* roots has significant effects on epithelial cell proliferation in human prostate cancer (10).

Acute lymphoblastic leukemia (ALL) is a category of leukemia as well as three other leukemia malignancies including acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL) and chronic myeloid leukemia (CML) (11). In ALL, different genetic and environmental factors result in malignant lymphoid cell growth. Origins of ALL are B and T cells (12). ALL is so common in older children and adults (13). To date, numerous plant anticancer agents have been discovered and developed in many laboratories (14). Some plant lectins with anti-leukemic effects have been found including abrin-a, lectin of Kurokawa mushroom, mistletoe lectins (ML-I, ML-II, ML-III) and VCA lectin (*Viscum album var. corolatum*) (15).

Small molecules are significant factors in cancer therapy, surely leukemia, due to targeting unsuitable regulation of transcription factors and decreasing cell contacts (13). Thus, finding new drugs with the best therapeutic effects are always needed to treatment leukemia. So, this study was designed to evaluate the anticancer effects of UDA in ALL because of therapeutic effects and very low molecular weight of UDA. Cytotoxicity of UDA on T and B leukemia cell lines and their responses to apoptosis are reported here.

Materials and Methods

Plant material

Stinging nettles were harvested from Tonekabon (Mazandaran, Iran) during winter and confirmed in the herbarium of Golestan Agricultural and Natural Resources Research and Education Center (herbarium number: 2541). Rhizomes were cut into small pieces after washing and used immediately or stored at -20ºC.

Purification of Urtica dioica agglutinin

UDA was purified as described by Peumanus *et al* (1984) (1). Briefly, rhizomes were homogenized in 0.1% HCl and extraction was carried out during several stages. Total UDA was purified from *Urtica dioica* extract by affinity chromatography on chitin. The column of chitin was equilibrated with acetate buffer and after passing the extract, unbinding proteins were washed off

with 1 M NaCl. Finally, the lectin desorbed with 0.5 N acetic acid. The lyophilized UDA was dialyzed against phosphate buffer saline (PBS).

Agglutination assay and analysis

The concentration of UDA was determined using lectin specific absorbance according to the method of Katiar *et al* (1999) (16). Monitoring UDA in isolation progress was performed by SDS-PAGE. Agglutination activity was assayed by the double dilution method in micro-tubes, in which 50 µl of 1.5 % suspension of human trypsin-treated erythrocytes was added to 50 µl of UDA solution. After 1-2 h incubation at room temperature, agglutination was determined macroscopically and microscopically (17, 18).

Cell lines

The cell lines including Jurkat (human T cell leukemia) and Raji (human B lymphocyte, Burkitt's lymphoma) were respectively purchased from Pasture Institute, Tehran, Iran, and Iranian Biological Resource Center, Tehran, Iran. The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS and penicillin-streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral whole blood using Ficoll-Paque media (Inno-train, Germany). 10 mLheparinized peripheral blood in A 50-mLtube was diluted with PBS (1:1). After mixing, the diluted blood carefully layered over the cold Ficoll- Paque. The tubes were centrifuged for 30 minutes at 1020 g. The interphase buffy coat layer was aspirated carefully. The cells were washed three times in warm PBS and resuspended in RPMI 1640 medium supplemented with penicillin-streptomycin and 0.5% FBS (19).

LDH assay

The early cytotoxicity of UDA was assessed using the Cytotoxicity Detection Kit (Roche, Cot. No. 11644793001) that analyzes the release of LDH (lactate dehydrogenase) into the culture medium. Jurakat and Raji cells were incubated with different concentrations (0, 0.5, 2, 8, 32, 128, 512 µg/mL) of UDA for 24 hours in 96-well plates. After mixing 50 µl of supernatant with 50 µl of the reaction mixture (catalyst and dye solution) and incubation at RT for 30 minutes, absorbance was recorded by a microplate reader (Bio Tek, USA) at 490 nm. The experiment was repeated three times in duplicates. Cells treated with 1% Triton X-100 had maximum LDH activity (high control) and untreated cells had minimum LDH activity (low control). The percentage of cytotoxicity was calculated according to the manufacturer's instruction using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{expriment value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Cell Growth, Apoptosis and Cell Cycle Analysis

Cell proliferation was reported based on 3-(4,5-dimethylthiazol-2yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra- zolium, inner salt (MTS) assay. 6×10^4 cells per well of Jurkat and 5×10^4 cells per well of Raji were seeded into a 96-well plate and treated with different concentrations of UDA and 0.5 µg/mL vincristine (positive control). Concentrations of UDA included 0, 0.5, 2, 8, 32, 128, 300 µg/mL for Jurkat cells and 0, 0.5, 2, 8, 32, 128, 450 µg/mL for Raji cells. After 72 h incubation in 37 °C, 10 µl of MTS reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit, Promega, USA) was added into each well and cells were incubated for 4 h. Plates were read using a microplate reader at 490 nm against 630 nm. The experiment was performed three times and IC_{50} (the concentration of the sample needed to inhibit 50% of cell growth relative to control) was determined. Cell growth (%) was shown as a ratio of absorbance in the sample (treated cells) relative to absorbance in control (untreated cells) (20).

$$\text{Cell growth (\%)} = \frac{\text{absorbance 490 nm (sample)}}{\text{absorbance 490 nm (control)}} \times 100$$

Fluorescent staining with Annexine V/PI (Biolegend, San Diego, CA) was used for the analysis of apoptosis induction. 3×10^5 cells per mL were seeded in 24 well-plates and treated by UDA at different concentrations (shown in figure 2A) for 72 h. The cells were stained according to the manufacture instruction. Then, apoptosis was analyzed by flow cytometry using BD Accuri™ C6 software.

For cell cycle analysis, 3×10^5 cells per mL (in 24 well-plates) were treated as stated for apoptosis assay, then harvested and fixed in 70% ethanol for 2 h at -20 ºC. Fixed cells were treated with RNaseA (Thermo Fisher) for 30 minutes at 37 ºC and stained with propidium iodide (Sigma), and finally, DNA content was measured by flow cytometry (BD Accuri™ C6 Flow Cytometer). The cell cycle phase distribution was analyzed by BD Accuri™ C6 software. All experiments were performed three times in duplicated and values were reported as mean ± standard error mean (SEM).

Western Blot

The cells (3 × 105 cells per mL in 6 well-plates) treated by UDA (at a concentration of 128 µg/mL) for 72 h were harvested and washed with PBS. Cell pellets were resuspended in lysis buffer (Urea 8 M, Thiourea 2 M, Tris 50 Mm (pH 7.4)), 1 mM PMSF and protease inhibitor cocktail (Roche) followed by incubation for 20 minutes at 4 ºC with a frequent vortex. The cell lysates were centrifuged at 13000 g for 20 minutes at 4 ºC. The protein concentration was determined by the Bradford method (21). The total protein was loaded on 12.5 % SDS polyacrylamide gel and transferred to microporous polyvinylidene difluoride (PVDF) membrane (Sigma). Then, membranes were blocked in PBS containing Tween 20 (0.5% in PBS) for 20 minutes at room temperature. Primary antibody incubation was performed overnight at 4 ºC under shaking for caspase 3, caspase 8, caspase 9 (1:300) and Beta-actine (control for the immunoblots) (1:500) (Santa Cruze). Membranes were washed thrice with PBS containing Tween 20 (0.05% in PBS). Thereafter, proteins were detected by HRP-conjugated secondary antibodies and visualized using 3,3′-diaminobenzidine (DAB, MP Biomedicals).

Statistical analysis

All graphs were prepared with GraphPad Prism 6.0 (GraphPad software). Values were expressed as mean ± SEM. Statistical analyses were performed using SPSS Version 21. The Kolmogorov-Smirnov test was used

to determine the normality of data. Differences among groups were analyzed using the Kruskal-Wallis test. The significant level was set to $p<0.05$.

Results

UDA purification

UDA isolated from rhizomes and roots of *Urtica dioica,* was analyzed by SDS-PAGE. One 8.5-9.5 kDa band was observed in 12.5 % SDS polyacrylamide gel (figure 1). The Purified UDA exhibited agglutination activity on human erythrocytes.

LDH assay and Cell Growth

The unwanted early cytotoxic effect of UDA by necrosis on lymphoblast leukemia cell lines, Jurkat and Raji, was determined by lactate dehydrogenase (LDH) assay after 24 h. Figure 2A showed the percentage of LDH increased just in high concentrations of UDA, especially in Jurkat cells. The 50% cytotoxic concentration (CC_{50}) value was 844.2 and 332.7 µg/mLin Jurkat and Raji cells, respectively.

Also, the cell growth of Jurkat and Raji treated with UDA was assayed by MTS during 48 and 72 h. Dose-dependent reduction in the cell survival was observed (Figure 2B) in comparison to a positive control (vincristine). Proliferation percentage in Jurkat cells treated with 0.5 µg/mL vincristine was 47.79% and 44.12% in 48 h and 72 h, respectively and these parameters in Raji cells were 56.47% and 49.21%, respectively. The proliferation of PBMC cells treated with UDA (128 µg/mL) for 48 h was not decreased. Table 1 presents the IC_{50} of UDA on Jurkat and Raji cells. These data indicated a time-dependent manner of UDA on cells because the IC_{50} values simultaneously decreased with increasing incubation time. Also, the UDA had stronger inhibition effect on Raji cells than Jurkat cells.

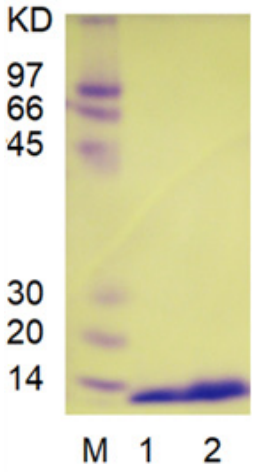

Figure 1. SDS-PAGE of UDA isolated from *Urtica dioia* in the presence (lane 1) and absence (lane 2) of reducing agent (2-ME). Lane M contains protein markers.

UDA causes apoptosis in Jurkat cells

To examine whether the cytotoxic effect of UDA on cells results in apoptosis, Jurkat and Raji cells were treated with different concentrations of UDA for 72 h, thereafter treated cells were stained by Annexine V/PI. As shown in figures 3A and 3B, changes in the early apoptotic population and late apoptotic/necrotic population of cells were remarkable just in Jurkat cells (at 128 µg/mLof UDA). Another cell line, Raji, and PBMC cells (as normal cells) did not show significant responses to UDA treatment.

Cell cycle analysis

To examine the mechanism of cell growth inhibition in the Jurkat and Raji cells treated with UDA (128 µg/ml), the percentages of the cell population in cell cycle phases were analyzed using flow cytometry. The results showed that treatment with UDA for 72 h increased the sub G1 phase to 36% in the Jurkat cells and 31.4% in the Raji cells (Figure 4 A and B). Significantly increased cancer cells in the sub G1 phase indicates UDA induced cell death.

Caspase 8, 9 and 3 activations in apoptotic Jurkat cells

To examine the apoptosis pathway in Jurkat cells, we assessed the activity of caspase 3, caspase 8 and caspase 9 with a specific monoclonal antibody against procaspase 3 (35 kD), procaspase 8 (57 kD) and caspase 9 (43 kD). Cells were exposed to UDA (128 µg/ml) and vincristine (0.5 µg/ml) as a positive control, for 72 h. We observed significant rising in the activity of caspase 3, caspase 8 and caspase 9 in cells treated with UDA and vincristine in comparison to control. Therefore, UDA induced apoptosis by both extrinsic and intrinsic caspase pathways.

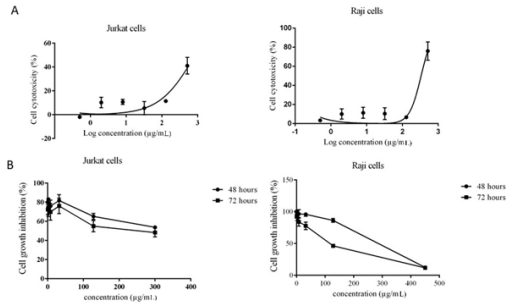

Figure 2. UDA inhibits the proliferation of Jurkat and Raji cells. (A) Jurkat and Raji cells were incubated with different doses of UDA (0, 0.5, 2, 8, 32, 128, 512 µg/mL) for 24 hours. LDH release assay was used for cell cytotoxicity evaluation. (B) Growth inhibition of Jurkat and Raji treated by UDA in different concentrations (0, 0.5, 2, 8, 32, 128, 300 µg/mL for Jurkat cells and 0, 0.5, 2, 8, 32, 128, 450 µg/mL for Raji cells) for 48 and 72 h. Cell growth inhibition was determined by the MTS assay.

Table 1. IC_{50} of UDA in Jurkat and Raji cells treated for 48 and 72 h.

Time/cell	IC_{50} (µg/mL) 95% Confidence Interval	
	Jurkat	Raji
48 h	259.0 (150.6- 445.4)	235.4 (199.8-277.3)
72 h	166.0 (78.8-349.8)	93.9 (67.3-131.2)

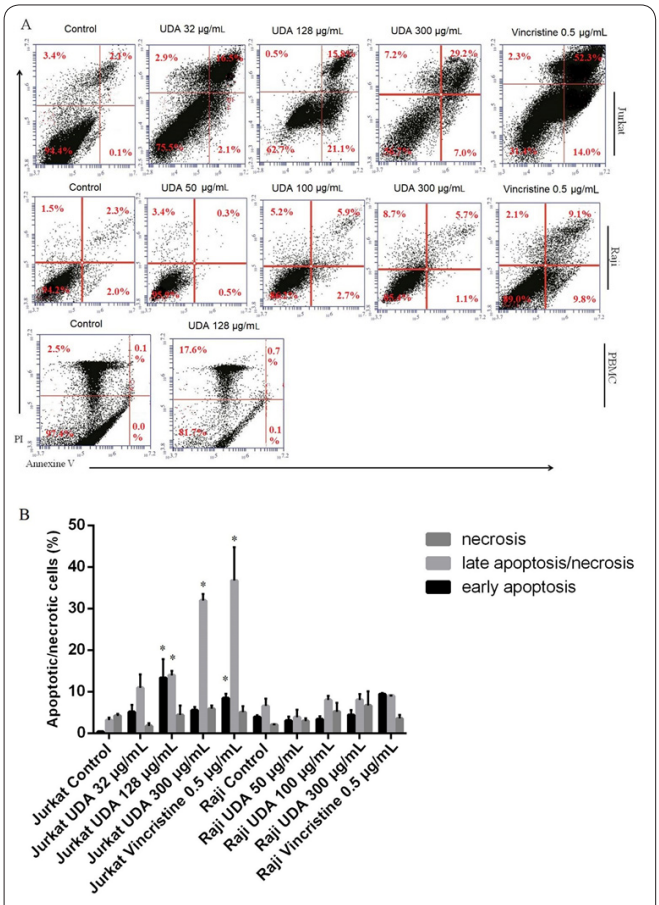

Figure 3. Apoptosis induction with UDA treatment in T-ALL cells. (A) Flow cytometry analysis of Jurkat and Raji cells treated with different concentrations of UDA for 72 hours. Viable (annexin V- PI-), early apoptotic (annexin V+ PI-), late apoptotic/necrotic (annexin V+ PI+) and necrotic (annexin V- PI+) cells were shown in representative figures. (B) The bar chart shows the values displayed in panel (A) that were mean ± SEM of three independent experiments. *P< 0.05.

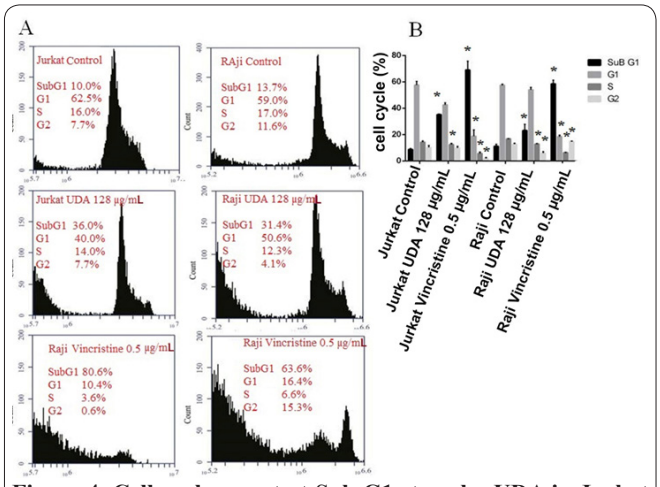

Figure 4. Cell cycle arrest at Sub G1 stage by UDA in Jurkat and Raji cells. (A) Jurkat and Raji cells were treated with 128 µg/mL UDA for 72 h. Cell cycle analysis was performed by flow cytometry. (B) Bar chart shows the data displayed in panel A. Values are mean ± SEM of three independent experiments. *P< 0.05.

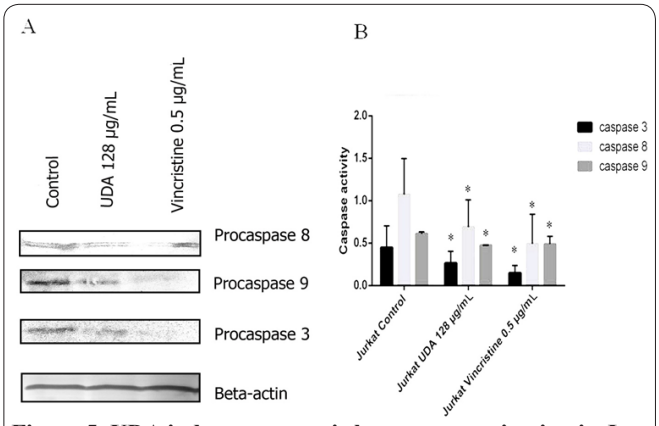

Figure 5. UDA induces apoptosis by caspase activation in Jurkat cells. (A) Immunoblot analysis of caspase 3, 9 and 8 in cells treated with 128 µg/mLUDA and 0.5 µg/mLvincristine (as a positive control) for 72 h and untreated cells (control). The expression of Beta-actin was used as an internal control. (B) Bar charts were quantified by densitometry. The values are reported as mean ± SEM of three independent experiments. *P< 0.05.

Discussion

Treatment of ALL in children and younger adults is more effective than elderly patients and leads to good results. ALL treatment in elderly patients is still a big challenge. Furthermore, the disease will relapse in most adult patients. Then, it is necessary to find new drugs for treating ALL and creating more therapeutic options (12). Here, we investigate the cytotoxic effect of stinging nettle lectin, UDA, on T and B cell lines (Jurkat and Raji). UDA inhibited Jurkat and Raji cell proliferation. The therapeutic effect of UDA on benign prostatic hyperplasia (BPH) was reported by Wagner *et al (*1995) that showed inhibition of A431 epidermal cancer cell membrane with UDA treatment (7).

In the following, results of flow cytometry showed higher apoptosis effect of UDA on Jurkat cells in comparison to Raji cells which exhibited a little apoptosis. In apoptosis, despite plasma membrane integrity, cell shrinkage and producing apoptotic bodies occurred. However, necrosis causes swelling in cells and destruction of the cell membrane (22).

Furthermore, the cell cycle analysis presented here shows DNA accumulation in the sub G1 phase for both Jurkat and Raji cells, while, in Raji cells, apoptosis was very low without a significant increase in necrotic cells. Kawabata *et al* (1999) reported a lack of caspase activation and resistance to apoptosis in Raji cells (23). In another study, Luciano *et al* (2002) compared apoptosis induction in T and B cell lines by measuring caspase activation and DNA fragmentation. DFF40 (caspase-activated DNAse) and DFF45 (inhibitor of caspase-activated DNAse) levels were determined, too. DFF40 is a mediator of DNA fragmentation. The results of these experiments indicated that DFF40 was undetectable in Raji cells and the ratio of DFF40 to DFF45 was very low. Hence, DNA fragmentation was not observed and Raji cells were resistant to apoptosis (24). Therefore, the response of Raji cells to apoptosis may be due to a defect in the apoptotic pathway or various models of cell death, such as autophagy. Autophagy is a type of programmed cell death (PCD) independent of caspases. In this pathway, autophagic vesicles are formed from cytoplasm mass and organelles and digested by lysosome (25). Then, further investigation would be required to determine the causes of UDA-induced death in Raji cells.

The activity of caspases 3, 8 and 9 were visualized by western blot to determine the response properties

associated with apoptosis in Jurkat cells. The activity of caspases, as endoproteases, depends on cysteine residues. Caspases are classified to the initiator (caspases 8 and 9) and executioner caspases (caspases 3, 6 and 7). Apoptosis starts by initiator caspases that activate executioner caspases affecting a cascade of molecules which results in cell death (26). The results of our experiments indicate an increase in the activity of both caspase 8 and caspase 9 and, also, changing the activity of caspase 3 with UDA treatment. Active caspase 8 starts apoptosis by activating executioner caspases in an extrinsic pathway and can induce apoptosis by the intrinsic pathway through affecting BID. Caspase 9 is an initiator in intrinsic apoptosis (26).

Most of the lectins such as ConA, ricin, *Rice bran* agglutinin (RBA) and *Polygonatum odoratum* lectin (POL) induce apoptosis in a caspase-dependent pathway. It is seen that caspase 8 plays a special role in most apoptosis induced with lectins (27). There are various mechanisms for apoptosis induction by lectins with anti-leukemic effects (15). Some studies have linked apoptosis to radiation (28-33) and genetics (34-36).

In conclusion, our work has led us to conclude that UDA induces apoptosis by extrinsic and intrinsic pathways in acute T-lymphoblastic leukemia cell line, Jurkat. However, UDA treatment results in a very low apoptosis effect on acute B-lymphoblastic leukemia cells, Raji. Therefore, UDA can be considered as a leukemia therapeutic agent for more studies. The mechanism of UDA-mediated cell death in Raji cells is not clear and needs further experiments.

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Interest conflict

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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