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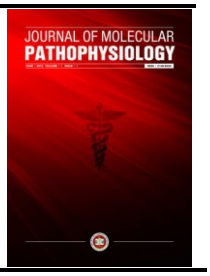


**Original Research**

# Cytotoxic effects of conjugated linoleic acids on human hepatoma cancer cells (HepG2)

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Conjugated linoleic acids; Human hepatoma cancer cells (HepG2); cytotoxicity, apoptosis; cell cycle

**Abstract**
Conjugated linoleic acids (CLAs) are group of polyunsaturated fatty acids that attract considerable attention due to their anticarcinogenic effects. In this study, the viability, apoptosis and cell cycle status of human hepatoma cells (HepG2) following treatment with *cis-9,trans-11(c*9,*t*11*)*, *trans*-10,*cis*-12 (*t*10,*c*12) and mixed isomers of CLA were investigated. Cells were grown routinely on RPMI 1640 media and treated with different concentrations of CLA isomers for 72 hours. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, acridine orange/propidium iodide (AO/PI) staining and terminal deoxynucleotide transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay were used to determine the results. The viability of HepG2 cells was reduced significantly ($P < 0.05$) by all CLA isomers tested in a dose-dependent manner. The median inhibitory concentration ($IC_{50}$) value varies with type of CLA isomer. Mixed isomers were significantly ($P < 0.05$) more potent than *c*9, *t*11 CLA isomer. All CLA isomers tested were able to induce characteristic apoptotic changes and significant ($P < 0.05$) proportion of apoptosis. Significantly ($P < 0.05$) higher proportion of cells in $G_{0/1}$ and lower proportion in $G_2$/M phases of the cell cycle in treated cells were also observed. Therefore, results suggested that CLA isomers reduce viability and proliferation of HepG2 cancer cells in relation with cell cycle arrest and induction of apoptosis.



## INTRODUCTION

Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of octadecadienoic (18:2) acid in which the double bonds are conjugated and occur at carbon atoms 7 and 9, 8 and 10, 9 and 11 or 10 and 12, or 11 and 13 with all possible *cis(c)* and *trans(t)* combinations [1]. Despite the possibility of various combinations of isomers, the *c*9,*t*11 is the main isomers found naturally in meat and dairy products of ruminants [1,2]. The second isomer which is commonly encountered is *t*10, *c*12 CLA [3, 4].

*In vivo* and *in vitro* studies have shown that CLAs have anticarcinogenic, antioxidant, antiatherosclerotic and antidiabetic effects [1-5]. Experimental models have been extended to include the positive role of CLAs on body composition, immune system and bone health [1, 2].

HepG2 is a cancer cell line derived from human hepatic carcinoma and widely used in *in vitro* studies [6]. The inhibitory effects of CLA on HepG2 cells were reported by Igarashi and Miyazawa [6] and Maggiora *et al.* [7]. However, most researches were conducted by using the mixture of *c*9,*t*11 and *t*10,*c*12 CLA isomers. There is a scarcity of reports on cytotoxic effects of

individual isomers. Indeed, the mechanism of action of CLA isomers is yet to be determined. Several mechanisms have been proposed including modulation of eicosanoid metabolism and signal transduction, anti-inflammatory activity, peroxisome proliferator-activated receptors activation [8, 9]. More recently, mechanism of action has been related with the induction of apoptosis [10, 11]. The ability to escape programmed cell death (apoptosis) is a hallmark of most cancer cells and often correlates with tumor aggressiveness and resistance to anticancer drugs [12]. Thus, the modulation of apoptosis has become an interesting target for both preventive and therapeutic approaches of cancer [13].

Loss of cell-cycle regulation is an important character of cancer cells. The cell cycle is controlled by intracellular concentration protein kinase which consists of a cyclin and a cyclin-dependent kinases. These proteins involved in DNA synthesis and mitosis, by acting on several check points in the cell cycle. The components involved in the progression of cells from one to the next phase of the cell cycle could also be important targets for anticancer agents [14]. Therefore, the objectives of this study were to assess the viability, the apoptosis and cell cycle status of HepG2 cancer cells following treatment with *c*9,*t*11, *t*10,*c*12 and mixed CLA isomers.

## MATERIALS AND METHODS

### Cell culturing

Hepatoma cancer cells (HepG2) were obtained from American Type Culture Collection. Cells were grown in RPMI 1640 media (Gibco® Invitrogen, Canada), that contain 100 U/mL penicillin (Gibco® Invitrogen, Canada), 100 µg/mL streptomycin (Gibco® Invitrogen, Canada) and 10% foetal bovine serum (Gibco® Invitrogen, Canada). Cells were routinely maintained and subcultured in 25 $cm^2$ plastic flasks at 37°C in a humidified $CO_2$ incubator (RS Biotech Laboratory Equipment Limited, UK) with 95% air and 5% $CO_2$. Twenty five percent of trypsin-EDTA (Gibco® Invitrogen, Canada) was used to detach cells from the bottom of the flask.

### MTT assay

The cytotoxic effects of CLA isomers were assessed using MTT assay. The assay is based the ability of viable cells to convert water soluble MTT reagent (tetrazolium salt) into a purple water insoluble formazan. By measuring spectrophotometrically the amount formazan produced, it is possible indirectly to know the amount of viable cells in the plate [15, 16].

Cells were seeded at density of $1x10^4$ cells per well in a 96 well plate. After overnight incubation, cells were treated with *c*9,*t*11 (purity ≥96 %) Cayman Chemical Ltd, USA) and *t*10,c12 (purity ≥98 %) (Cayman Chemical Ltd, USA) and mixed (42% *c*9,*t*11, 44% *t*10,*c*12; about 10% *c*10,*c*12 and 5% of a mixture of others) (Sigma chemical Co., USA) CLA isomers, and 5-fluorouracil (Sigma chemical Co., USA) at concentration of 5, 10, 20, 40, 80, 120 and 180 µg/mL. Serum free RPMI 1640 media was used to dilute and obtain the treatment concentrations. After 72 hours of incubation, 10 µL of MTT labelling reagent (Invitrogen™ Limited, UK) was added into each well. The plates were then incubated again for 4 hours. After this incubation period, excess MTT reagent was aspirated and 50 µL of dimethyl sulphoxide (Sigma chemical Co., USA) was added to each well and mixed thoroughly. The plate was then transferred to microplate reader (Opsys MR™, Dynex Magellan Biosciences Company, USA) and absorbance was recorded at 540 nm. Each treatment at different concentrations and the untreated control were in three wells, and the experiment was repeated at least three times.

### Acridine Orange/Propidium Iodide Staining

The morphological changes of cells treated with CLA isomers and 5-fluorouracil were observed using acridine orange/propidium iodide (AO/PI) staining. The media containing about $2x10^5$ cells was transferred to a $25cm^2$ flask and incubated overnight. Then, cells were treated with CLA isomers and 5-fluorouracil, and incubated for 72 hours. The concentration for treatment was based on the $IC_{50}$ value from MTT assay. After the incubation period, the cells were harvested, washed twice with phosphate buffer saline (Gibco® Invitrogen, Canada) and resuspended with 10µL of AO (100 µg/mL) (Sigma chemical Co., USA) and 10 µL PI (100 µg/mL) (Sigma chemical Co., USA). About 10 µL of the suspended fluid was transferred on to a microscope slide and viewed under the fluorescent microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

### TUNEL Assay

Tunel assay was conducted to assess and quantify the induction of apoptosis and cell cycle arrest by CLA isomers. The media containing about $2x10^5$ cells was transferred to a $25cm^2$ flask and incubated overnight. Then, cells were treated with CLA isomers and 5-fluorouracil, and incubated for 72 hours. The concentration of the treatment was based on the $IC_{50}$ value from MTT assay. Then, cells were harvested and washed twice with PBS. Five mL of one percent (w/v) paraformaldehyde (Sigma chemical Co., USA) in PBS was added and placed on ice for 15 minutes. The cells were centrifuged at 1000 rpm for 10 minutes. The cells were washed and centrifuged twice by adding 5 mL of PBS. The cells were added to 5 mL of ice cold 70% (v/v) ethanol (Sigma chemical Co., USA). Then, the

cells were kept for a minimum of 30 minutes on ice or in a -20°C freezer before the continuation of the next procedure.

Cells in ethanol were placed in 12 × 75mm flow cytometry centrifuge tubes. The cell suspension was centrifuged at 1000 rpm for 10 minutes and ethanol was removed by aspiration. Each tube was resuspended with one mL of washing buffer (Invitrogen™ Limited, UK) and centrifuged for 10 minutes at 1000 rpm. This step was repeated twice. Cell pellets of each tube were resuspended with 50µL of the DNA labelling solution (Invitrogen™ Limited, UK). The cells were incubated in the DNA labelling solution for 60 minutes at 37°C in a temperature controlled bath. Cells were shaken every 15 minutes to keep the cells in suspension. At the end of the incubation time, one mL of rinse buffer (Invitrogen™ Limited, UK) was added to each tube and centrifuged at 1000 rpm for 10 minutes. The cell rinsing was repeated once more by adding one mL of rinse buffer to each tube and centrifuged at same speed and time. Cell pellets were resuspended in 100 µL of antibody solution (Invitrogen™ Limited, UK) and left at room temperature for 30 minutes in dark. Half mL of propidium iodide/RNase a staining buffer (Invitrogen™ Limited, UK) was added to each sample and left for 30 minutes at room temperature in dark. Finally, samples were analyzed by flow cytometry (DakoCytomation, Denmark) and histograms were analyzed by Summit V4.3 software. Each treatment was repeated at least three times.

### Statistical Analysis

Data were expressed as mean with their respective standard deviation and differences among treated groups were assessed using one way analysis of variance followed by Duncan's multiple range test.

## RESULTS

### Cytotoxic Effects

Figure 1 shows the percentage viability of HepG2 cells following treatment with different concentrations of CLA isomers and 5-flurouracil (positive control).

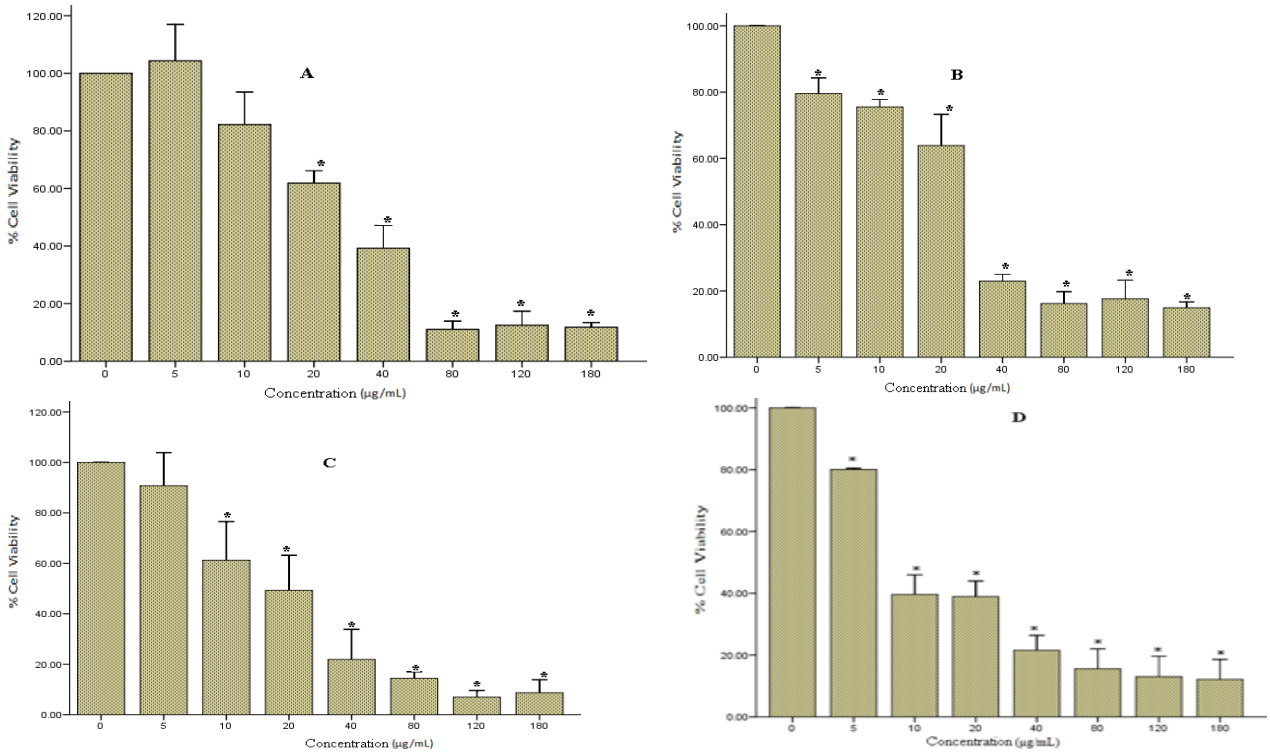


**Fig.1:** Viability of HepG2 cancer cells following treatment with CLA isomers and 5-flurouracil for 72 hours (Percent (%) cell viability is expressed as mean (n=3) percentage of untreated control values; *significantly different ($P$<0.05) from untreated control

A: Percent viability of cells treated with *c*9,*t*11 CLA isomer
B: Percent viability of cells treated with *t*10,*c*12 CLA isomer
C: Percent viability of cells treated with mixed CLA isomers
D: Percent viability of cells treated with 5-fluorouracil

The viability cells was significantly ($P$<0.05) reduced by all CLA isomers used in a dose-dependent manner. Significant ($P$<0.05) reduction in cell viability was observed at a concentration as low as 5 µg/mL for *t*10, *c*12; 10 µg/mL for mixed and 20 µg/mL for *c*9,*t*11 isomers of CLA. The median inhibitory concentrations ($IC_{50}$) for mixed, *t*10,*c*12 and *c*9,*t*11 CLA isomers were 15.40 ±6.6, 25.65 ±5.4 and 31.79 ±5.1 µg/mL, respectively. Comparison of these values indicated that mixed isomers were significantly ($P$<0.05) more potent than *c*9,*t*11 CLA isomer.

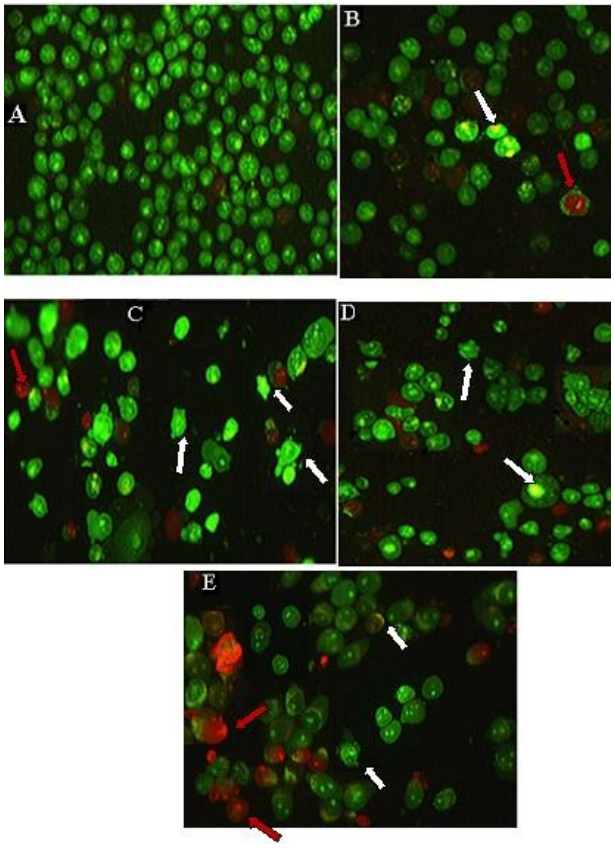


**Fig 2:** HepG2 cells stained with AO/PI following treatment with CLA isomers and 5-fluorouracil at their respective $IC_{50}$ for 72 hours as viewed under fluorescent microscope (x200 magnification).
A: Control untreated cells with almost uniform colour and shape
B, C, D and E: cells treated with *c*9,*t*11; *t*10,*c*12 and mixed CLA isomers, and 5-fluorouracil, respectively; showing more proportion of cells undergoing apoptosis (white arrows) and late apoptotic /apoptotic bodies or necrotic cells (red arrows)

### Apoptosis Induction and Cell Cycle Arrest

CLA isomers have shown cytotoxicity effect on HepG2 cancer cells. However, their mechanisms of action remain to be investigated. One of the proposed mechanisms of action has been related with induction of programmed cell death (apoptosis) [10, 11]. In order to assess the induction of apoptosis by CLA isomers, AO/PI staining and TUNEL assay were conducted.

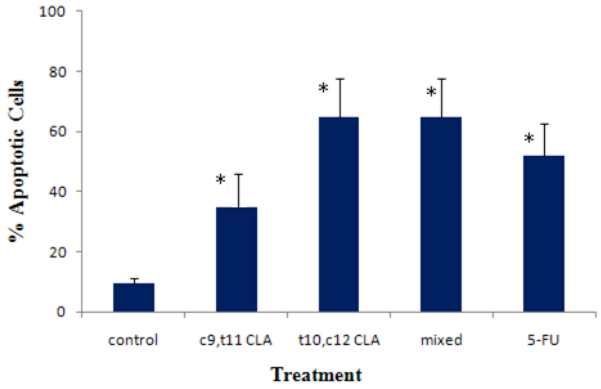


**Fig 3:** Percentage of apoptotic HepG2 cells following treatment with CLA isomers and 5-fluorouracil (5-FU) at their respective $IC_{50}$ for 72 hours (Values are mean (n=3) ±SD) (* denotes significant ($P$ < 0.05) difference compared to the untreated control)

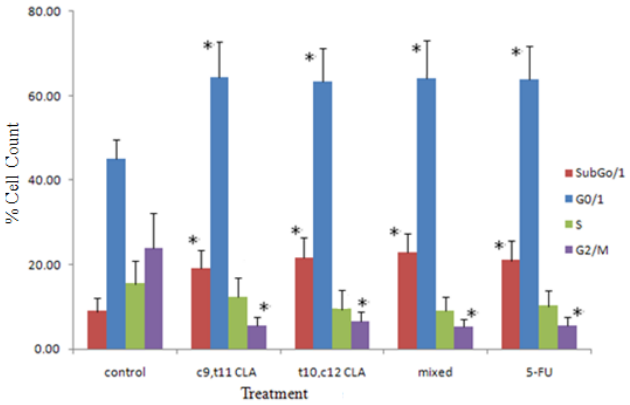


**Fig 4:** Cell cycle phase status of HepG2 cells following treatment with CLA isomers and 5-fluorouracil (5-FU) at their respective $IC_{50}$ for 72 hours
(Values are mean (n=3) ± SD) (*denotes significant ($P$<0.05) difference compared to the untreated control)

Figure 2 depicts the colour and morphological changes of AO/PI stained HepG2 cancer cells after treatment of cells with CLA isomers and 5-fluorouracil for 72 hours. Most untreated control cells were stained green whereas treated cells stained orange indicating that membranes were damaged and permeable to PI. In treated cells, red staining was also observed showing free entrance of PI into apoptotic bodies and necrotic

cells. In addition to the colour difference between treated and untreated cells, there were also differences in size and appearance of the cells. Unlike untreated cells, the size and appearance of treated cells were not uniform showing shrinkage, ruffling and blebbing of the membrane.

The TUNEL assay was based on the most important character of apoptosis particularly the fragmented DNA in which DNA fragments enzymatically incorporate 5-bromo-2′-deoxyuridine 5′-triphosphate at 3'-OH end broken DNA [17]. The reaction can be quantified by flow cytometry which gives the proportional percentage of apoptotic and non-apoptotic cells. Figure 3 shows the mean percentage of apoptotic cells. All treatments induced significantly ($P$<0.05) higher proportion of apoptotic cells than untreated control. There was no significant difference between the treatments in induction of apoptosis.

The technique used to display Figure 3 shows only the proportion of apoptotic cells. It did not show any correlation with cell cycle phases. Using DNA content analysis, it was possible to get the proportion of cells in different phases of the cell cycle. The mean proportion of HepG2 cells at different phases of the cell cycle is as depicted in Figure 4. Apoptotic cells appear in sub$G_{0/1}$ peak as a consequence of partial DNA loss [18]. The proportion of apoptotic cells (in sub$G_{0/1}$) was significantly *($P$ < 0.05)* higher in treated than untreated cells. Significantly ($P$<0.05) higher proportion of cells in $G_{0/1}$ phase and lower proportion of cells in $G_2$/M phase in treated was also observed. There was no significant difference in the proportion of cells in S phase in both treated and untreated groups. There was no significant difference in proportion of cells in different phases among treatments. These results indicated that CLA isomers block the progression of HepG2 cells form $G_{0/1}$ phase to the next thereby reduce the cell proliferation.

## DISCUSSION

This study was conducted to assess and compare the viability of HepG2 cancer cells following treatment with different isomers of CLA and to explore more whether the cytotoxic effect is related with apoptosis and cell cycle. The viability of HepG2 cancer cells was investigated using MTT assay and the results revealed that CLA isomers reduced cells viability in a dose-dependent manner. Inhibitory effect of CLA isomers on HepG2 cells was also reported by Igarashi and Miyazawa [6]. According to their report, HepG2 cell proliferation was inhibited when cells treated with the mixture of *c*9,*t*11 and *t*10,*c*12 CLA isomers for 72 hours in a dose-dependent manner.

In this study, it was observed that that antiproliferative activity of individual CLA isomers was varied. Mixed isomers show better inhibitory effects than *c*9,*t*11 CLA isomer. The stronger effects of mixed isomers could be related with the presence of most potent isomers in it or it may be due to their additive or synergistic effect in mixed form.

It has been frequently reported that defective apoptotic pathways as the major causative factor in the development and progression of cancer [17, 19]. Cells undergoing apoptosis exhibit a series of characteristic morphological and biochemical changes including cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [17, 20]. Two techniques (AO/PI staining and TUNEL assay) were used in this study to assess apoptotic features in treated cells. AO/PI staining was based on the membrane integrity and incorporation of these dyes with cellular DNA. AO and PI intercalate with DNA once inside the cell, and emit green and red fluorescence, respectively. PI doesn't enter into viable cells with intact membrane so only viable cells will take only AO and appear green in fluorescent microscope. Non-viable cells will take both dyes and appear orange to red due to the dominance of PI [21]. Morphological investigations using this staining confirmed that cells showed typical characteristics of apoptosis such as cell shrinkage, membrane blebbing and ruffling when treated with CLA isomers. In treated groups, there were large number of cells stained orange to red colour of PI due to membrane damage but untreated cells were almost uniform in size and shape, and emit green fluorescence of AO. Although AO/PI staining correlate with other apoptotic assessment techniques, the staining is not as such specific since it can stain cells with membrane damage other than apoptosis. Therefore, to confirm apoptosis a more specific TUNEL assay was conducted.

TUNEL assay explores DNA fragmentation which is an important biochemical hallmark of apoptosis. DNA fragmentation yields large number of 3-OH group that serve as starting points for terminal deoxynucleotidyl transferase (TdT) enzymatic reaction (17, 22]. Addition of the deoxythymidine analog 5-bromo-2′-deoxyuridine 5′-triphosphate to the TdT reaction serves to label the break sites. These labelled DNA are attached with fluorescein antibody which can be quantified by flow cytometry [20, 22]. Flow cytometric analysis showed the ability of CLA isomers to induce apoptosis on HepG2 cancer cells. These will provide evidences to elucidate the probable mechanism of action of CLA isomers in relation to the induction of apoptosis. This was also reported by Park et al. [10], Belury [11] and Hsu and Ip [23].

Induction of tumour cell death by cell cycle arrest is the most common mechanism of action of various

anticancer agents [14]. Conjugated linoleic acid isomers elicited cell cycle arrest in $G_{0/1}$ as it was seen that proportion of HepG2 cells in $G_{0/1}$ phase of the cell cycle was significantly ($P<0.05$) greater than that of the untreated control. When cell progression is blocked, the probability to follow apoptotic pathway is extremely very high.

In conclusion, the results of the present study showed that CLA isomers have inhibitory effects on the proliferation of HepG2 cancer cells which is related with cell cycle arrest and induction of apoptosis.

## ACKNOWLEDGMENTS

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