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In vivo anticancer activity of vanillin, benzophenone and acetophenone thiosemicarbazones on Swiss albino mice

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PEER REVIEW

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Comments

This is a good study in which the authors determined the *in vivo* antitumor property of vanillin thiosemicarbazone, benzophenone thiosemicarbazone and acetophenone thiosemicarbazone against transplantable tumor EAC cells and normal peritoneal cells. This study demonstrated that these molecules can inhibit *in vivo* the proliferation of EAC cells and extend the lifespan of induced mice.

Details on Page 815

ABSTRACT

Objective: To study the anticancer activities of three schiff bases *viz.* vanillin thiosemicarbazone, benzophenone thiosemicarbazone and acetophenone thiosemicarbazone against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice.

Methods: Synthesized compounds have administrated into the intraperitoneal cavity of the EAC inoculated mice at two doses. The anticancer activities have studied by monitoring the parameters such as cell growth inhibition, tumor weight measurement, survival time of EAC bearing mice as well as the changes in depleted hematological parameters due to tumorigenesis. All such data have been compared with those of a known standard drug bleomycin at the dose of 0.3 mg/kg (*i.p.*).

Results: It has been found that these bases enhanced life span, reduced average tumor weight and inhibited tumor cell growth of EAC cell bearing mice remarkably. The results were similar in potency to those obtained with bleomycin. It was also found that the depleted hematological parameters (red blood count, white blood count and haemoglobin content) were found to be restored gradually towards normal within few weeks after ceasing the treatment.

Conclusions: The compounds can be primarily considered more or less as potent anticancer agents.

KEYWORDS

Anticancer activity, Ehrlich ascites carcinoma cells, Thiosemicarbazones

1. Introduction

Schiff bases are the most widely studied biological active compounds in recent times. Schiff bases alone and their metal complexes have a wide range of anticancer, antibacterial, antifungal, anti-inflammatory, anti-tubercular, analgesic and pesticidal activities^[1–8]. In our laboratory schiff bases

like N-(1-phenyl-1-hydroxy-2-phenyl-ethylidene)-2'-hydroxyphenylimine^[5], acetone semicarbazone^[9], vanillin semicarbazone^[10], benzophenone semicarbazone^[11] *etc.* have been used to study their anticancer activities. They all are found to be potent anticancer agents. Not only the schiff bases, schiff base complexes with transition metals have also been investigated against Ehrlich ascites carcinoma (EAC) cells in

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Swiss albino mice^[4,12]. In the present paper three schiff bases, namely, vanillin thiosemicarbazone (VTS), benzophenone thiosemicarbazone (BTS) and acetophenone thiosemicarbazone (ATS) have been selected as test compounds and their anticancer activity against EAC cells *in vivo* was studied. In support of this work, hematological studies have also been done accordingly.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

2.2. Experimental animal

Swiss albino mice of 5–7 weeks old, weighing 20–26 grams were used as experimental animals. They were collected from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B), Mohakhali, Dhaka.

2.3. Animal care

Mice were kept in iron cages with saw dust and straw bedding which were changed once a week regularly, standard mouse diet (recommended and prepared by ICDDR'B) and water were given in adequate.

2.4. Synthesis of the compounds

The compounds were synthesized by the method in the same way as described in literature^[13,14].

2.5. Characteristics

The synthesized compounds were verified by taking melting points (Table 1), and conducting infrared spectral (IR) studies^[13–15]. The new bond $-C=N-$ (azomethine) formed during the synthesis in all the cases was confirmed from IR spectra at around 1630 cm^{-1} which was in this accordance with the literature^[15].

Table 1

Physical constants of the synthesized schiff base compounds.

Compounds	LD ₅₀ (mg/kg)	Melting point (°C)	Color	Solubility
VTS	55	136–138	White	DMSO, Ethanol
BTS	16	169–170	White	DMSO, Ethanol
ATS	20	114–116	White	DMSO, Ethanol

2.6. Tumor cells

Transplantable tumor EAC cells were used in this experiment. The initial inoculums of EAC cells were kindly provided by Indian Institute of Chemical Biology (IICB), Kolkata, India. The EAC cells were thereafter propagated in our laboratory biweekly through intraperitoneal (*i.p.*) injections of 3×10^6 cells per mouse.

2.7. Determination of median lethal dose (LD₅₀)

The test compounds were separately dissolved in 2% dimethylsulfoxide and injected intraperitoneally to six groups of mice (each group containing six animals) with different

doses. The LD₅₀ values were estimated from the plots of number of mortality versus dose curve^[16].

2.8. Study of anticancer activities

The procedures followed for the anticancer activities of the compounds were similar to those described elsewhere^[11,16].

2.8.1. Cell growth inhibition

Eight groups of mice (six in each group) were used for the experiment for each of the compounds. In every mouse, 136×10^4 EAC cells were inoculated into each group on Day 0. Treatments were started after 24 h for tumor inoculation and continued for five days. Group 1 and 2 received VTS at the doses of 5.0 and 7.5 mg/kg (*i.p.*), Group 3 and 4 were treated by BTS at the doses of 1 and 2 mg/kg (*i.p.*) and Group 5 and 6 were treated by ATS at the same doses like BTS per day per mouse. Group 7 received bleomycin at the dose of 0.3 mg/kg (*i.p.*) and Group 8 was used as control. The doses so used were far below the LD₅₀ values of the compounds and considered here as their effective doses for the experiment. Mice in each group were sacrificed on Day 6 and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypan blue and then counted by a hemocytometer. Total numbers of viable cells in every animal of the treated groups were compared with those of control (EAC treated only) group.

2.8.2. Average tumor weight and mean survival time (MST)

Eight groups of mice (six in each group) were used for the experiment for test compounds. EAC cells 136×10^4 were inoculated in each mouse on Day 0. Treatment was started after 24 h of tumor cell inoculation and continued for 10 d. The weight changes of each mouse were recorded daily and the increase in tumor weight was monitored. The host survival time was recorded and expressed as mean of survival time in days. The percent increase of life span (ILS %) was calculated by using the following formulae:

$$\text{MST} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{total number of mice}}$$

$$\text{ILS\%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100$$

2.9. Hematological parameters in normal and tumor bearing mice

The effect of the test compounds on hematological parameters was studied in both normal and tumor bearing mice. For tumor bearing mice, treatment was started after 24 h of EAC cell transplantation and continued for 10 d. For normal mice, the same procedure was followed. Blood was drawn out (from tail vein) from each group of mice on Day 5, 10, 15 and 25 and then the following parameters were studied by the methods as described by Ruisa and Sood^[17].

2.9.1. Estimation of percentage of hemoglobin

The amount of hemoglobin was measured by using Sahli's hemometer. Blood was drawn out with the aid of a micropipette (up to the desired mark) from mouse and transferred to the cuvette (tube) in hemometer containing a little amount of 0.1 mol/L HCl solution. Distilled water was added and stirred until a good color match was obtained by the standard color prism of the hemometer. The reading of the solution in the cuvette was noted. From the cuvette reading, hemoglobin content in g/dL was calculated.

2.9.2. Total red blood cell (RBC) count

Exactly 10 µL non-coagulated blood was drawn out with the tip of a micropipette and diluted to 1000 times with red cell counting fluid (Tri-sodium citrate 8%, formaldehyde 37%, distilled water 55%) and mixed properly. The resultant mixture was checked in Neubauer hemocytometer and the number of cells was counted with a microscope. Total RBC per mL were calculated.

2.9.3. Total white blood cell (WBC) count

Non-coagulated blood of 10 µL was diluted with 1 mL WBC counting fluid (aqueous acetic acid solution 2% v/v, aqueous methylene blue 0.3% w/v) and mixed properly. The dilution factor was 100. Total WBC was counted with hemocytometer like that of RBC counting technique.

2.10. Normal peritoneal cells

Seven groups of normal mice (six in each) were treated with the test compounds at the doses of 1 mg/kg (i.p.) and 2 mg/kg (i.p.) for both BTS and ATS, again 5 mg/kg (i.p.) and 7.5 mg/kg (i.p.) for VTS for three consecutive days. The untreated group (Group 7) was used as control. On the fourth day, the animals were sacrificed after injecting 5 mL of normal saline (0.98%) into the peritoneal cavity of each mouse. Intraperitoneal exuded cells and number of macrophages were counted with 1% neutral red by hemocytometer¹⁸.

2.11. Statistical analysis

All values were expressed as mean±standard error of mean (SEM). Statistical analysis was performed with One way analysis of variance (ANOVA) followed by Dunnett's 't' test using SPSS statistical software of 14 version. P<0.05 was considered to be statistically significant when compared with control.

3. Results

The results of the experiments as described in the previous sections have been presented here. In most cases, average values of repeated experiments have been taken. Lethal doses of VTS, BTS and ATS have been found to be 55 mg/kg, 16 mg/kg and 20 mg/kg respectively for intraperitoneal treatment in mice. Effects of the test compounds and bleomycin on the tumor weight due to tumorigenesis are shown in Figures 1 and 2. Treatment of the animals with the test compounds, previously inoculated with EAC cells, resulted in the inhibition of tumor growth pronouncedly. The

effects of test compounds and bleomycin on EAC cell growth on Day 6 after tumor transplantation have been shown in Table 2. Treatment with VTS, BTS and ATS resulted in cell growth inhibition at 7.5 mg/kg for VTS, 2 mg/kg for both BTS and ATS doses to be 88.04%, 83.86% and 88.97% respectively. Bleomycin at the dose of 0.3 mg/kg (i.p.) showed 88.50% cell growth inhibition.

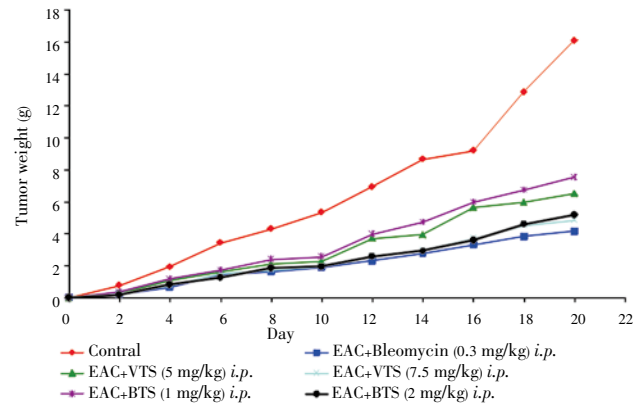


Figure 1. Effect of VTS and BTS on average tumor weight in mice (mean±SEM) (n=6).

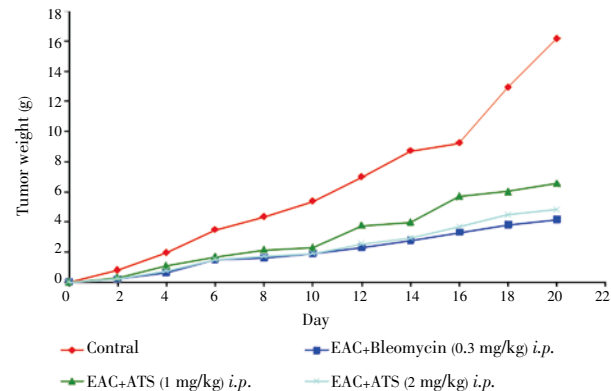


Figure 2. Effect of ATS on average tumor weight in mice (mean±SEM) (n=6).

The effects of test compounds on survival time at different doses have also been summarized in Table 3. It was observed that life span increased significantly in tumor induced mice after treatment with the test compounds. Treatment with VTS, BTS and ATS at doses 7.5 mg/kg, 2.0 mg/kg and 2.0 mg/kg respectively increased life span by 86.67%, 80.95% and 88.09% respectively as compared to that of control mice. The survival time was found to be increased with increased doses. Bleomycin at the dose of 0.3 mg/kg (i.p.) increased life

Table 2

Effect of schiff bases on EAC cell growth inhibition (in vivo) (mean±SEM, n=6).

Test compounds	Nature of the drug	Dose (mg/kg)	No. of EAC cells in mice on Day 6 after tumor cell inoculation	Cell growth inhibition (%)
Control (EAC cell bearing mice)	–	–	(6.275±0.034)×10 ⁷	–
EAC+Bleomycin	Antibiotic	0.3	(0.721±0.005)×10 ⁷ ****	88.50
EAC+VTS	Synthetic	5	(1.325±0.009)×10 ⁷ ****	78.88
		7.5	(0.750±0.006)×10 ⁷ ***	88.04
EAC+BTS	Synthetic	1	(1.94±0.005)×10 ⁷ ****	69.08
		2	(1.013±0.006)×10 ⁷ ****	83.86
EAC+ATS	Synthetic	1	(1.38±0.009)×10 ⁷ ***	78.01
		2	(0.692±0.006)×10 ⁷ ****	88.97

Treatment days=5; **P<0.01, and ***P<0.001 between control and treated groups.

Table 3

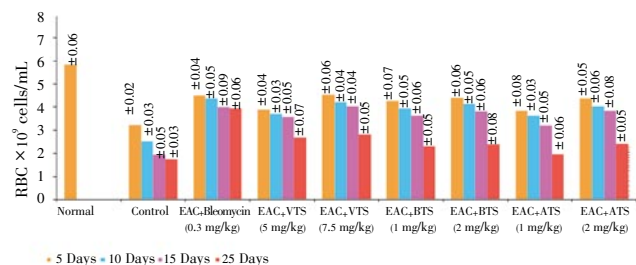
Effect of schiff bases on survival time of EAC cell bearing mice (mean±SEM) (n=6).

Treatment	Nature of the drug	Dose (mg/kg, i.p.)	Mean survival time (days)	% Increase of life span
Control (EAC cell bearing mice)	–	–	21.0±0.78	–
EAC+Bleomycin	Antibiotic	0.3	40.0±0.64***	90.48
EAC+VTS	Synthetic	5	34.8±0.45*	65.71
		7.5	39.2±0.68***	86.67
EAC+BTS	Synthetic	1	32.6±0.85**	55.24
		2	38.0±1.05***	80.95
EAC+ATS	Synthetic	1	34.6±0.88***	64.76
		2	39.5±0.76**	88.09

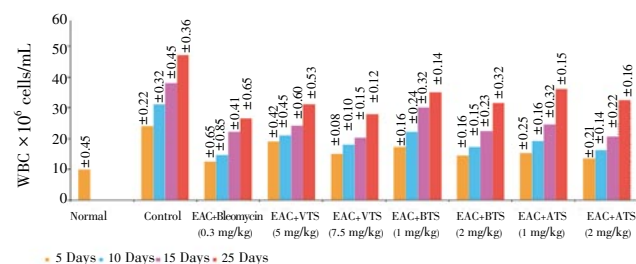
Treatment days=10; *P<0.05, **P<0.01, and ***P<0.001 between control and treated groups.

span by 90.48% when compared to control.

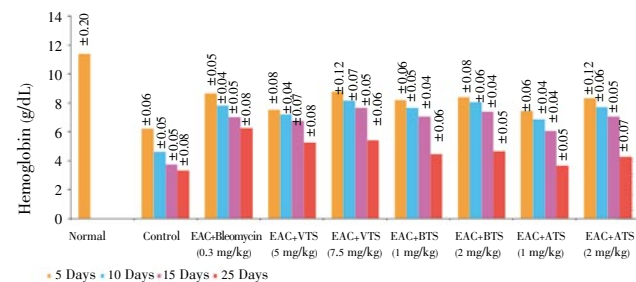
The effects of the test compounds on the hematological parameters of the tumor bearing mice are shown in Figures 3 to 5. These parameters varied from their normal values along with tumor growth. The hemoglobin content and RBC counts decreased, whereas the WBC counts increased after the inoculation of EAC cells. After treatment with the compounds, the parameters were found to be restored moderately.

**Figure 3.** Effect of test compounds on RBC of EAC cells bearing mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

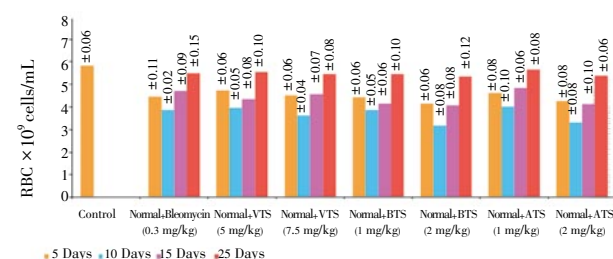
**Figure 4.** Effect of test compounds on WBC of EAC cells bearing mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

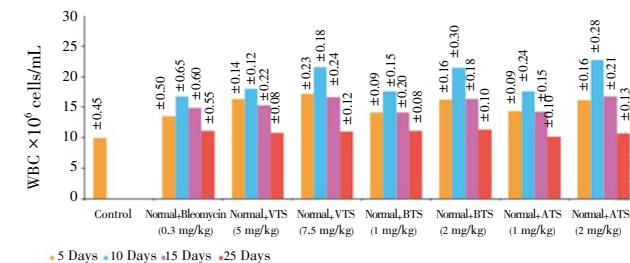
**Figure 5.** Effect of test compounds on hemoglobin of EAC cells bearing mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

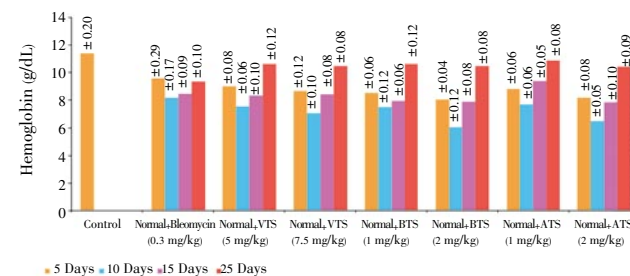
The effects of the test compounds on the hematological parameters of the normal mice have been shown in Figures 6 to 8. The test compounds showed low toxicity to the host during the treatment period, but these parameters almost restored back to normal values within 25 d.

**Figure 6.** Effect of test compounds on RBC of normal mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

**Figure 7.** Effect of test compounds on WBC of normal mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

**Figure 8.** Effect of test compounds on hemoglobin of normal mice on days 5, 10, 15 and 25.

Treatment time: 10 days; Number of mice in each group: 6.

The effects of test compounds on peritoneal cells in normal mice at different doses have been shown in Table 4. These bases noticeably enhanced the number of peritoneal macrophages.

Table 4

Effect of schiff bases on the enhancement of peritoneal cells in mice (mean±SEM, n=6).

Treatment	Dose (mg/kg, <i>i.p.</i>)	Macrophages per mL	Total peritoneal cells× 10 ⁶ per mL
Control (Normal)	–	1.65±0.26	9.25±0.32
Normal+VTS	5	1.80±0.21***	9.60±0.08**
Normal+BTS	1	1.72±0.22**	9.85±0.18**
Normal+ATS	1	1.94±0.32*	9.90±0.32*

Treatment days=3; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ between control and treated groups.

4. Discussion

The potency of the compounds as anticancer agent has been judged by measuring (i) reduction of average tumor weight, (ii) cell growth inhibition and (iii) enhancement of life span of the EAC cell bearing mice. The efficiency of the compound has been compared with the data obtained by running parallel experiments with a known effective anticancer drug, bleomycin at the dose of 0.3 mg/kg and also with those obtained with similar type of compounds available in the literature^[10,11].

For EAC cell bearing mice, the tumor weight has been found to increase rapidly. The treatment of such mice with test compounds reduced the cell growth rate. The results showed that the treatment with the compounds inhibited cell growth efficiently. VTS at the dose of 7.5 mg/kg (*i.p.*) inhibited cell growth by 88.04%, BTS and ATS both with 2 mg/kg (*i.p.*) inhibited cell growth by 83.86% and 88.97% respectively.

The life span of the EAC cell bearing mice increased remarkably when treated with the test compounds. The data are also comparable to those found in the literature^[10,11]. The prolongation of the life span of cancer bearing mice is a very important and reliable criterion for judging the potency of any drug as anticancer agent^[19]. In effect, the results are quite comparable to those obtained with other schiff bases and also with bleomycin. The positive effect of the compounds against EAC cell bearing mice has further been verified by monitoring the change in hematological and biological parameters. The RBC and hemoglobin contents of EAC cells bearing mice decrease gradually with time when compared to those of normal mice. The reduction of both RBC levels and hemoglobin percentage is the major problem in cancer bearing animals. The problems, which are usually encountered in cancer chemotherapy are myelosuppression and anemia. This is probably owing to the deficiency of iron in hemolytic or myelopathic condition^[20,21]. Treatment with the test compounds probably reversed back RBC levels and hemoglobin contents towards normal as the reduction rates were found to be slowed down in comparison to those of EAC bearing untreated mice. With the growth of tumor, WBC level increases with time. The rise of WBC count of the EAC bearing

treated mice followed much slower rate in comparison with that of EAC bearing untreated mice. Parallel hematological experiments have been done with normal mice to evaluate the host toxicity effect of the test compounds. A very slight deterioration of such parameters has been observed during the treatment period (25 consecutive days). Similar toxic effect has also been observed^[16] with bleomycin at the dose of 0.3 mg/kg (*i.p.*).

The immunological effect of the test compounds in fresh healthy mice has been performed by counting peritoneal macrophages which has provided further support for the potency of the test compounds as anticancer agents. The test compounds have noticeably enhanced the number of macrophages. This enhancement might have produced some cytokinetic products, such as tumor necrosis factor, interleukins, interferons, *etc.* which in turn may be responsible in destroying tumor cells^[22].

In conclusion, the test compounds showed pronounced efficiency against EAC cells in Swiss albino mice. Important characteristics *viz.* cell growth inhibition, tumor weight reduction and the enhancement of survival time of EAC cell bearing mice with test compounds. The results are quite comparable to those obtained with other schiff bases and bleomycin. The data may serve as valuable information for advanced cancer researches in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The search for anticancer drugs from natural and synthetic origin increases with time. Several studies have shown that positive results *in vitro* are not conclusive *in vivo* due to several factors. However, some studies have shown excellent anticancer activity *in vivo* with plant extracts, improved formulations or synthetic molecules.

Research frontiers

This research work demonstrated the *in vivo* anticancer properties of three synthetic molecules VTS, BTS, ATS against

EAC cells at different concentrations for 25 d with bleomycin (0.3 mg/kg body weight) used as positive control. The results showed that these molecules act in different manners on physical, hematological and lifespan of the mice.

Related reports

The data about the greater number of *in vivo* anticancer activity of vanillin thiosemicarbazone, benzophenone thiosemicarbazone on EAC cells have been demonstrated by SMM Ali *et al.* (2012, *Asian Pac J Trop Biomed*) and Islam K *et al.* (2012, *Cancer Biol Med*). The same parameters have been carried out in these studies and the only difference was found on the concentration treatment (concentration of different molecules).

Innovations and breakthroughs

Data regarding the *in vivo* anticancer property of acetophenone thiosemicarbazone on EAC cells are scarce. This study has showed that ATS inhibited EAC cell bearing mice up to 88% and increased the lifespan of the mice in the same percentage.

Peer review

This is a good study in which the authors determined the *in vivo* antitumor property of vanillin thiosemicarbazone, benzophenone thiosemicarbazone and acetophenone thiosemicarbazone against transplantable tumor EAC cells and normal peritoneal cells. This study demonstrated that these molecules can inhibit *in vivo* the proliferation of EAC cells and extend the lifespan of induced mice.

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