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Carcinogenesis induced by neonatal exposure to various doses of 5-bromo-2′-deoxyuridine in rats

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Abstract

Male and female outbred LIO rats were exposed to subcutaneous injections of 3.2 mg 5-bromo-2′-deoxyuridine (BrdUrd), on day 1; days 1 and 3; or days 1, 3, 7, and 21 following birth. The mean life-span decreased by 40.9, 31.2 and 38.9% in males exposed to 1, 2 or 4 injections of BrdUrd, respectively, and by 22.9, 10.5 and 23.4% in females, respectively, compared to the controls. The agent increased the population aging rate of the exposed male and female rats. Total tumor incidences in males exposed to 0, 3.2, 6.4 or 12.8 mg of BrdUrd were 21.1, 27.3, 40.0 and 33%, respectively, and in the females 44.3, 54.5, 48.1 and 56.3%, respectively. Only the largest dose of BrdUrd significantly increased the tumor incidence in the exposed rats as compared to the control ones ($P < 0.05$). However, tumor latency was shorter in any group of rats exposed to BrdUrd, as compared to the control one. The exposure to 0, 1, 2 or 4 injections of BrdUrd was followed by the development of testicular Leydigomas in 0, 0, 28 and 12% of males, respectively. There was no organ or tissue target for BrdUrd in the females. However, the development of tumours not typical for the rats and the widening of tumor spectrum was observed in the female rats exposed to the agent.

Keywords: 5-Bromo-2′-deoxyuridine; Dose-response; Carcinogenesis; Rat

1. Introduction

5-Bromo-2′-deoxyuridine (BrdUrd), a thymidine analog, can substitute for thymidine in DNA and induce many of the biological effects both in vitro and in vivo [1]. Thus, it has been reported both to enhance [30] and to inhibit [39] cell differentiation, and inhibit DNA synthesis and cell replication in vivo [36] and in vitro [24].

As it has been shown, BrdUrd is mutagenic in cellular systems [12,34]. It produces second generation mutants in the eukaryotic algae *Volvox cartesi* [18], and it also appears to have a miscoding effect in cell-free systems [32], being incorporated into the replicating DNA in the place of thymidine. BrdUrd may occur in not only the usual keto form but also in an enoltautomeric

Abbreviations: BrdUrd, 5-Bromo-2′-deoxyuridine.

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form [11], which forms hydrogen bonds with the guanine instead of adenine, normal pair for thymidine and 5-bromouracil. Assuming that there is no 5-bromouracil repair in rat DNA [20,21], and that BrdUrd after incorporation into BrdUrd pairs with guanine when present as the enol tautomer, base pair substitution mutations are expected to occur (GC → AT and AT → GC transitions) during the subsequent DNA replication [9]. Two of 3 mesenchymal tumors developed in rats exposed neonatally to BrdUrd contained a GGT → GAT codon 12 point mutation [7]. A comprehensive review on the genetic toxicology of BrdUrd in mammalian cells has been published recently [23].

It was shown in our previous experiments that neonatal exposure of rats to BrdUrd was followed by a slight carcinogenic effect in rats and in mice [2,25]. This paper presents the results of a study on the dose-response carcinogenic effect of the neonatal exposure of rats to BrdUrd.

2. Materials and methods

2.1. Chemicals

BrdUrd, from Sigma Chemical Co. (USA), 100% pure, was stored at 4°C.

2.2. Animals

Outbred LIO male and female rats from the animal department at N.N. Petrov Research Institute of Oncology [3] were used in this study. After mating and detection of pregnancy, they were kept 1 per polypropylene cage until delivery. The offspring were kept with the dam for 4–5 weeks in housing conditions. After that, 6–7 animals were held per cage. They received standard laboratory chow [4] and tap water ad libitum.

2.3. Experiment

After delivery, we randomly subdivided each dam and litter into 4 groups. BrdUdr was ex tempore dissolved in the distilled water and injected subcutaneously (0.1 ml) into the rats on day 1, or on days 1 and 3, or on days 1, 3, 7 and 21 of life, at a single dose of 3.2 mg per animal (groups 2–4). Thus, the total doses of BrdUrd per animal were 3.2, 6.4 or 12.8 mg in groups 2–4, respectively. Some rats born at the same time as the rats from group 4 were injected with 0.1 ml of the solvent and served as the controls (group 1). Animals were allowed to die naturally or were killed when moribund.

2.4. Pathohistological examination

Complete necropsies were performed on all animals. All organs were carefully examined macroscopically according to the recommendations of IARC [22] and Devor et al. [10]. The contexts of observations were registered for all tumors and were evaluated as 'fatal' or 'incidental' according to the IARC recommendations [13]. At the autopsy, all organs with tumors or with lesions that raised a suspicion of tumor growth as well as the liver, kidney and spleen were fixed in 10% neutral formalin. Then, following routine histological treatment, they were embedded in paraffin. Sections, 5–7-μm thick, were stained with haematoxylin and eosin. The tumors were classified according to the IARC classification [30].

2.5. Statistics

Experimental results were statistically processed according to the IARC recommendations [14]. The statistical procedure included non-parametric analysis of survival curves and of tumor-free survival curves. with computation of log-rank statistics and combined analysis of fatal and incidental tumor data using CARTEST comuter program [14]. In other places, Student's t-test and Fischer's exact test for equality of proportions were used [16]. An IBM PC/AT computer (USA) was employed for the statistical processing of the obtained data.

3. Results

The data presented in Tables 1 and 2 show that the administration of BrdUrd in the early life is followed by the significant decrease of the mean and maximum life-span of both male and female LIO rats. However, this life-span reduction did not depend upon the dose of the agent. The significant shift of the survival curves to the left was observed in the groups of male and female rats exposed to any dose of BrdUrd in the early stage of life, as compared to untreated animals (Figs. 1,2). The

Table 1
Survival, tumor incidence, localization and type in male rats neonatally exposed to various doses of BrdUrd

Parameters	Control	BrdUrd (mg)		
		3.2	6.4	12.8
Number of rats	71	14	32	85
Mean life-span, days[a]	779 ± 16	460 ± 56	536 ± 35	633 ± 19
Maximum life-span, days	1095	883	862	910
Age at the 1st tumor detection, days				
Any tumor	369	475	216	252
Fatal tumor	786	475	261	295
Number of tumor-bearing rats:				
Total (%)	15 (21%)	4 (29%)	10 (31%)	25 (29%)
Malignant (%)	4 (6%)	2 (14%)	2 (6%)	12 (14%)
Number of tumors				
Total	20	7	17	35
Malignant	4	2	2	12
Tumor site and type				
Pituitary				
Adenoma	9	1	1	4
Adenocarcinoma	—	1	1	1
Thyroid				
Adenoma	3	1	3	7 (5)[b]
Testis				
Leydigoma	—	1	9 (7)	10 (9)
Prostata				
Adenoma	—	—	—	1
Haemopoietic system				
Lympholeukemia	1	1	—	2
Thymus				
Malignant thymoma	1	—	—	2
Kidney				
Adenoma	1	—	—	—
Adenocarcinoma	1	—	—	—
Mesenchymal tumor	—	—	—	3
Lung				
Adenoma	1	—	—	—
Liver				
Malignant hemangiopericytoma	—	—	1	—
Colon				
Adenocarcinoma	—	—	—	2
Adrenal gland				
Cortical adenoma	—	—	2	—
Mammary gland				
Fibroma and fibroadenoma	2	2	—	1
Soft tissues				
Malignant fibrous histiocytoma	1	—	—	2

[a]Given as mean ± S.E.M. [b]Number in parentheses, number of rats. [c]The difference with controls is significant, $P < 0.001$.

aging rates of the populations of rats exposed to 4 injections of BrdUrd and those of the untreated ones were calculated according to Gompertz equation: $R = R_o \exp(at)$, where R = mortality; R = mortality at time (t) = 0; a = constant, and were evaluated as the left part of equation: $\Delta R/\Delta t = R_o \cdot a \cdot \exp(at)$ [14]. The calculations showed the increase in the parameter both in males

Table 2
Survival, tumor incidence, localization and type in female rats neonatally exposed to various doses of BrdUrd

Parameters	Control	BrdUrd (mg)		
		3.2	6.4	12.8
Number of rats	79	13	29	107
Mean life-span, days[a]	722 ± 15	557 ± 55	646 ± 34	569 ± 15
Maximum life-span, days	1013	846	874	853
Age at the 1st tumor detection, days				
Any tumor	402	293	421	159
Fatal tumor	557	293	421	263
Number of tumor-bearing rats:				
Total (%)	35 (44%)	7 (54%)	13 (45%)	58 (54%)
Malignant (%)	5 (6%)	5 (38%)	2 (7%)	15 (14%)
Number of tumors				
Total	63	14	29	89
Malignant	5	5	2	15
Tumor site and type				
Pituitary				
Adenoma	18	2	7	20
Adenocarcinoma	—	1	1	—
Thyroid				
Adenoma	3	2	5	11
Mammary gland				
Fibroma and fibroadenoma	33 (22)[b]	5 (3)	13 (11)	34 (27)
Malignant fibrous histiocytoma	—	—	—	1
Ovary				
Thecoma	1	—	—	1
Uterus				
Endometrial Polyp	—	—	2	3
Adenocarcinoma	1	—	—	1
Adenosarcoma	—	—	—	1
Haemopoietic system				
Lympholeukemia	1	1	—	3
Myeloleukemia	1	—	—	1
Thymus				
Malignant thymoma	1	—	—	—
Kidney				
Mesenchymal tumor	1	2	—	3
Mesodermal tumor	—	—	1	—
Salivary gland				
Adenoma	—	—	—	1
Liver				
Cholangioma	—	—	—	1
Pancreas				
Adenocarcinoma	—	—	—	1
Stomach				
Polyp	—	—	—	1
Colon				
Adenocarcinoma	—	—	—	1
Adrenal gland				
Cortical adenoma	3	—	—	—
Zymbal gland				
Squamous-cell carcinoma	—	—	—	1
Skin				
Papilloma	—	—	—	1
Soft tissues				
Fibroma	—	—	—	1
Malignant fibrous histiocytoma	—	1	—	2

[a]Given as mean ± S.E.M. [b]Number in parentheses, number of rats. a,b,c, The difference with controls is significant. (a) $P < 0.01$; (b) $P < 0.05$; (c) $P < 0.001$.

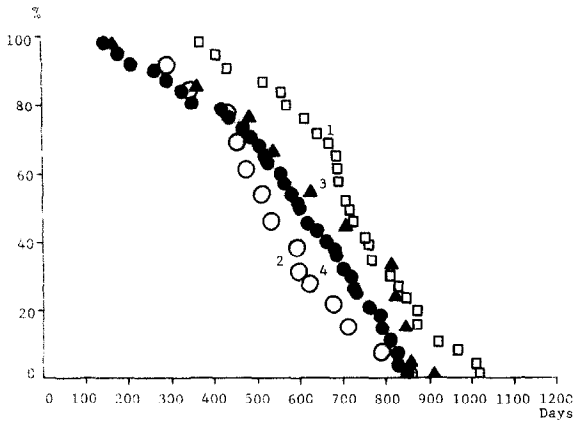

Fig. 1. Survival of male rats exposed to various doses of BrdUrd. Y axis, number of rats (%); X axis, age (days). 1, control; 2, BrdUrd 3.2 mg; 3, BrdUrd 6.4 mg; 4, BrdUrd 12. 8 mg. The symbols represent each third animal in groups 1, 3 and 4, and each animal in group 2.

(0.0229 ± 0.00074 days^{-2} and 0.0049 ± 0.00033 days^{-2}, respectively) and in females (0.0024 ± 0.00077 days^{-2} and 0.0047 ± 0.00021 days^{-2}, respectively).

Both male and female rats exposed to BrduRd manifested a delay in body weight gain in comparison to controls. The body weight of animals treated with BrdUrd was lower than in sex-matched controls during the whole period of observation. The estimated difference in body weight of BrdUrd-treated and control rats between 6–18 months of age exceeded 50 g in males and 30 g in females. Detailed data on this matter were published elsewhere [2].

The non-tumorous lesions of internal organs, mostly kidney, have also been observed in a number of rats neonatally treated with various doses of BrdUrd. Thus, the relative size of kidneys was sometimes decreased in these rats compared to the control ones. Histologically, the convoluted tubules in the kidneys of rats exposed to BrdUrd were enlarged and dilated to form micro- and macrocysts and were lined with eosinophilic epithelial cells which either tightly filled the lumen of tubules or protruded into it. These cystic lesions were observed in 4.2, 7.1, 9.3 and 21.2% ($P < 0.001$) of males and in 2.5, 15.4 ($P < 0.05$) 24.1 ($P < 0.01$) and 55.7% ($P < 0.001$) of females exposed to 0, 3.2, 6.4 or 12.8 mg of BrdUrd, respectively. The cases of decrease of the relative size of the liver histologically manifesting spongiosis, and decrease of the spleen size accompanied by the declines in the size of follicles and fibrosis of

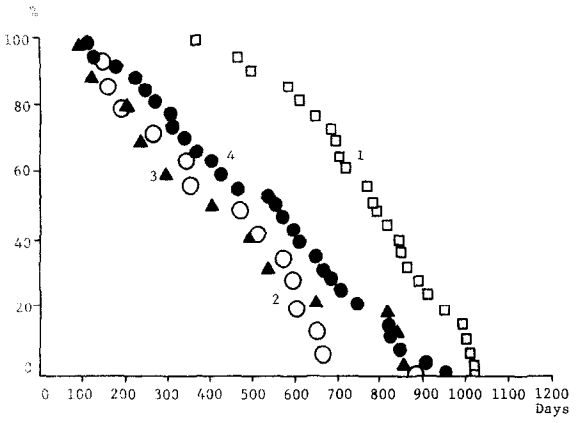

Fig. 2. Survival of female rats exposed to various doses of BrdUrd. Y axis, number of rats (%); X axis, age (days). 1, control; 2, BrdUrd 3.2 mg; 3, BrdUrd 6.4 mg; 4, BrdUrd, 12.8 mg. The symbols represent each third animal in groups 1, 3 and 4, and each animal in group 2.

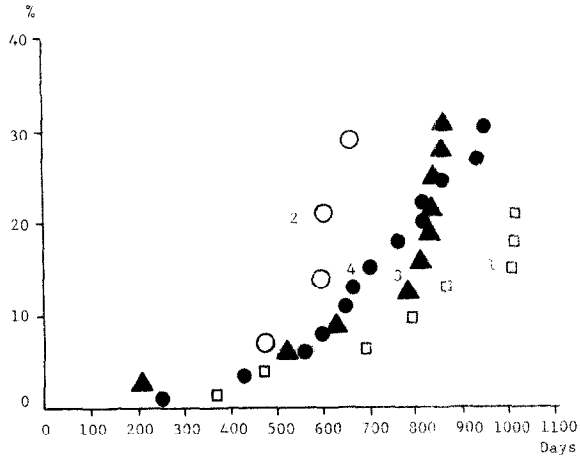

Fig. 3. Total tumor yield in male rats exposed to various doses of BrdUrd. Y axis, number of tumor-bearing rats (%); X axis, age (days). 1, control; 2, BrdUrd 3.2 mg; 3, BrdUrd 6.4 mg; 4, BrdUrd 12.8 mg. The symbols represent each second tumor-bearing animal in groups 1 and 4, and each animal in groups 2 and 3.

stroma, and atrophy of testicles have also been registered in rats exposed to BrdUrd.

The exposure to 12.8 mg of BrdUrd was followed by a significant increase in the incidence of both total and fatal tumors, as compared to the untreated controls. One or 2 injections of BrdUrd failed to influence the total tumor incidence in male and female rats significantly. However, the tumor yield curves were significantly shifted to the left in the groups of males and females exposed to any dose of BrdUrd, as compared to the controls (Figs. 3,4). We failed to observe any statistically significant difference in the total tumor incidence between the groups of rats exposed to various doses of BrdUrd (Tables 1,2).

Testicular Leydigomas were found only in males exposed to BrdUrd and the incidence of these tumors was significantly increased in the rats exposed to 6.4 and 12.8 mg of BrdUrd as compared to the ones exposed to 3.2 mg of the agent ($P < 0.05$). Mesenchymal tumors of kidney and colon adenocarcinomas were also discovered in the males exposed to the largest dose of BrdUrd and were not observed in the ones treated with the smaller doses of the agent.

The incidence of thyroid adenomas was slightly increased in the females exposed to BrdUrd (group 3 vs. controls, $P < 0.05$). The incidence of mesenchymal tumors of kidney was increased ($P < 0.05$) in the females treated with 3.2 mg BrdUrd, as compared to the controls. Endometrial polyps were discovered only in the BrdUrd-exposed females. The spectrum of tumors developed in the females treated with the largest dose of BrdUrd was significantly widened as compared to any other group.

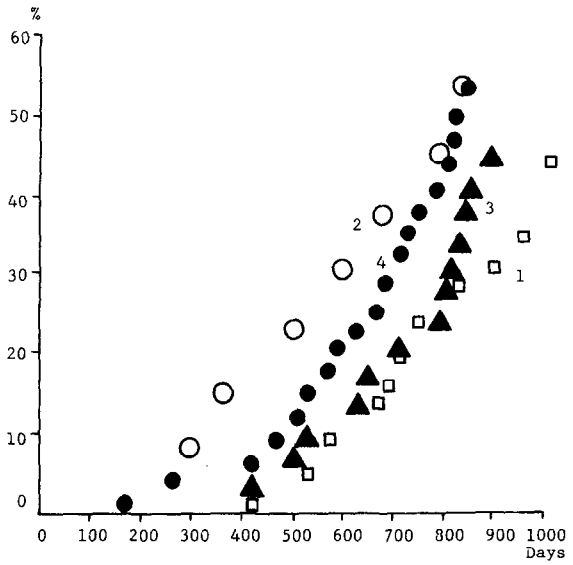

Fig. 4. Total tumor yield in female rats exposed to various doses of BrdUrd. Y axis, number of tumor-bearing rats (%); X axis, age (days). 1, control; 2, BrdUrd 3.2 mg; 3, BrdUrd 6.4 mg; 4, BrdUrd 12.8 mg. The symbols represent each third tumor-bearing animal in groups 1 and 4, and each animal in groups 2 and 3.

4. Discussion

As it was observed in our experiment, neonatal administration of BrdUrd to rats was followed by reduction of their mean life-span and acceleration of the aging rate (Tables 1,2; Figs. 1,2). The mean life-span was decreased by 40.9, 31.2 and 18.7% in the male rats and by 22.9, 10.5 and 21.2% of the females exposed to 3.2, 6.4 and 12.8 mg of BrdUrd, respectively, in comparison to controls. In general, our data do not contradict those reported by Craddock [8]. The mean survival of rats exposed neonatally to 1.6, 3.2, 6.4 or 12.8 mg of BrdUrd was 90.3, 55.3, 63.9 and 50.3 weeks, respectively, in that experiment [8]. There were only 6–15 animals per group and no data on the sex-adjusted survival rates, as well as the data on the survival of the controls, were reported.

Calculation of the mortality parameters for both untreated rats and those exposed to neonatal administration of BrdUrd, that survived to the age of 3 months, revealed (according to the Gompertz equation) an acceleration of the aging rate under the influence of the thymidine analogue. The results of monitoring of estrous function suggested the acceleration of natural age-related switching-off of reproductive function in female rats neonatally exposed to BrdUrd [2]. Long-term supplementation of the nutrient medium with BrdUrd also shortened the life-span of *D. melanogaster* and sensitized them to the life-span reducing effect of irradiation in the doses of 125–500 Gy [27]. Analysis of the character of survival curves of the

population allowed the authors to suggest that DNA is a primary substrate of the observed shortening of the life-span of the insects and that it is similar to natural aging.

In comparison with the DNA changes having a high miscoding potential, such as O6-alkylguanine or O4-alkylthymine [26] the presence of 5-bromouracil in the DNA results in a very low probability of miscoding [9,17]. However, in contrast to the alkylated bases that undergo the intensive repair [26], 5-bromouracil is not a substrate for the DNA repair enzymes and thus it persists over a long period of time [21,35]. It was shown in vitro that BrdUrd-induced mutagenesis in mammalian cells was dependent upon the perturbation of endogenous deoxycytidine metabolism, that mutations could arise from the misincorporation of BrdUrd into DNA, driven by the unbalanced deoxynucleoside triphosphate pools available for DNA synthesis, and that mutagenesis under these conditions resulted primarily in the GC → AT transitions [9,17]. Using an in vitro shuttle vector system, Davidson et al. [9] have revealed a very high degree of sequence specificity for the BrdUrd mutagenesis. Thus, BrdUrd-induced GC → AT transitions occurred almost exclusively in the sequences with the 2 adjacent guanine residues, and in about 90% of the cases the guanine residue involved in the mutation was the one in the more 3′ position.

It was revealed that BrdUrd considerably increased the number of chromosomal aberrations in the cells and sister chromatid exchange in vitro [19,28] and in vivo [38]. In our experiment, neonatal exposure of rats to 12.8 mg of BrdUrd was followed by the 2-fold increase in the number of chromosomal aberrations in the peripheral blood lymphocytes at the age of 3 months [2]. This was in agreement with the aforementioned data and also suggested the mutagenic potential of BrdUrd in vivo.

Does the life-scan reduction induced by BrdUrd appear because of its specific effect on the aging process or is it a result of the agents toxicity? Upon administration to the adult mice or rats, BrdUrd, unincorporated into DNA, is rapidly driven out of cells and undergoes catabolism to generate uracil and bromidic ion in the liver [23]. It is difficult to deny any early and late toxic effect of neonatal administration of BrdUrd in the rats which could reduce their life-span. Thus, neonatal exposure of rats to 4 injections of BrdUrd at a single dose of 3.2 mg per animal was followed by an increased mortality from the age of one week up to weeks 2–3 of life, and the mortality rate was decreased to the control level 1–1.5 weeks after the last injection of BrdUrd was made, i.e. after the period of persistence in the body of one of the products of its degradation — bromine [2].

A delay of differentiation and development of microcystic lesions in salivary glands, as well as a delay in postnatal development of testes and weakening of spermatogenesis was observed during the first 2 weeks of life in rats subjected to neonatal treatment with BrdUrd [5,13]. The development of macro and microcysts in the kidney registered in the previous studies [2,25] and the dependency of this pathology on the dose of BrdUrd observed in the present work, could be attributed to the effect of BrdUrd on the differentiation of kidney cells. A long-term persistence of the neonatally-administered BrdUrd in renal cortex DNA and in kidney DNA was shown in the same model [21,35]. The mesenchymal kidney tumors developed in rats neonatally exposed to BrdUrd [2,25, present data] expressed the point mutations in the Ki-*ras* protooncogene [7]. The fact is that some of these effects could depend on the mutagenic potential of the nucleoside analogue.

Assuming a fairly even level of BrdUrd incorporation into the DNA of various tissues of neonatal rats and long-term persistence in them [21,35], those cell populations with the most proliferative activity will be more likely to undergo malignant transformation. Since exposure to BrdUrd has been shown to have dramatic effects on cellular functions, including cell differentiation, inactivation of a regulatory genes or master switch [17,31] and proliferation [36], they may be important for any chronic influence on specific sensitive tissues and cells. In the present study, as well as in the reported earlier ones [2,25], neonatal administration of BrdUrd was followed by a slight increase in total tumor incidence, by the shortening of both the mean life-scan and the tumor laten-

cy and by the loss of body weight. These data do not permit the suggestion that the carcinogenic effect of the exposure to BrdUrd might be a consequence of its influences on body weight and longevity. It is well known that exceeded body weight is directly correlated with the incidence of spontaneous tumors and indirectly correlated with longevity [29,37].

We failed to observe a dose-response dependency of the effect of BrdUrd on the total tumor incidence, but tumor yield curves of both males and females exposed to 12.8 mg of the agent were shifted to the left, as compared to those for rats exposed to 6.4 mg of BrdUrd. As for the tumor yield curves for rats exposed to 3.2 mg of BrdUrd, it is possible that their shifting is more occasional and could depend on the small number of animals in this group.

The majority of the tumors developed in rats exposed to the various doses of BrdUrd, were presented by the spontaneous neoplasias characteristic for the rat strain used. However, tumors of gonads, kidney, uterus and some other localizations which were very seldom or never observed in the untreated LIO rats [3] were also discovered following autopsy of BrdUrd-treated rats. These data provide additional evidence to the conclusion [1,2,25] that a sole perturbation of DNA contributed substantially not only to the induction of cell transformation in vitro [6], but also to the carcinogenesis in vivo.

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