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Effects of LS 1727, a Nitrosocarbamate of 19-Nortestosterone, on Dimethylbenz(a)anthracene-Induced Mammary Tumors in the Rat

R. BOUVENG, M. ELLMAN, P. O. GUNNARSSON, G. JENSEN, J. LILJEKVIST and J. MÜNTZING*

Research Laboratories, AB Leo, S-251 00 Helsingborg, Sweden

Abstract—*LS 1727, a nitrosocarbamate of 19-nortestosterone, caused a dose-dependant retardation of growth of dimethylbenz(a)anthracene-induced mammary tumors in rats. LS 1727 had a more pronounced depressive effect on tumor growth and tumor DNA synthesis than 19-nortestosterone or CCNU. The uterine weight was increased and the ovarian weight decreased by LS 1727. The uptake of radioactivity in the tumors after injection of ^{3}H-dihydrotestosterone was reduced by LS 1727 but not the radioactivity uptake after injection of ^{3}H-estradiol-17β or ^{3}H-progesterone. There was no accumulation of radioactivity in the tumors after injection of ^{3}H-LS 1727 labeled in the steroid moiety but the radioactivity concentration declined with time parallel with the decline in blood concentration. Thin layer chromatography revealed that LS 1727 is rapidly metabolized to 19-nortestosterone and polar metabolites. It is concluded that LS 1727 may have a cytostatic effect on the tumors owing to an action of the alkylating moiety of the compound. The cytostatic action may be enhanced by androgenic and estrogenic effects of the compound.*

INTRODUCTION

MAMMARY tumors in rat and man have been shown to have receptors for estrogen and to be able to accumulate exogenous estrogen [1–7]. This characteristic may be a prerequisite for tumor growth inhibition achieved by estrogen [8, 9] and estrogen-linked cytostatic agents [10]. Mammary tumor growth in rat and man is affected also by androgen [8, 11–13], and receptors for androgen have been demonstrated in mammary tumors [7, 14]. Therefore it seemed worthwhile to examine the effects of LS 1727, a nitrosocarbamate of 19-nortestosterone (Fig. 1), on rat mammary tumors induced by dimethylbenz(a)anthracene (DMBA). LS 1727 has a cytostatic effect on experimental tumors such as L1210 Leukemia and Hepatoma AH130 as well as an androgenic effect [15]. Thus this compound might be expected to affect DMBA-induced mammary tumors both through a cytostatic action of the compound and through an effect obtained by the hormonal action of the drug.

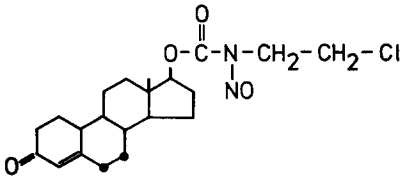

Fig. 1. Structural formula of LS 1727 including the positions of ^{3}H (●).

The primary aim of the present study was to examine the effect of LS 1727 on tumor growth. To elucidate the mode of action of the compound we also examined the effect of LS 1727 on steroid hormone uptake in the tumors as well as the uptake and metabolism of LS 1727 in the tumors.

MATERIALS AND METHODS

The animals used in this study were virgin female Sprague–Dawley rats obtained from AB Anticimex, Sollentuna, Sweden. They were maintained at 23°C on a 12-hr light and 12-hr dark cycle. All animals had free access to drinking water and food pellets

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*Send all correspondence concerning this manuscript to: Dr. J. Müntzing, AB Leo, S-251 00 Helsingborg, Sweden.

(Anticimex pellets No. 213). When 50 days old the animals were given a single oral dose of 20 mg DMBA (Eastman Kodac Co., Rochester, N.Y. 14650) dissolved in 1 ml of sesame oil. Beginning about 7 weeks later the animals were examined twice weekly and the average diameter (the mean of the longest diameter and the one perpendicular to this) for each tumor was registered. Animals with 1–5 tumors were selected for the experiments. Each animal was required to have at least 1 growing tumor with an average diameter larger than 10 mm.

LS 1727 was synthetized at AB Leo, Helsingborg, Sweden. 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) was obtained from H. Lundbeck & Co. A/S, Copenhagen, Denmark and 19-nortestosterone from Diosynth B.V., Oss, the Netherlands. Other nonradioactive chemicals and reagents used were of highest available purity and were used without further purification. LS 1727, [6, 7-^{3}H], (Fig. 1), specfic activity 16 mCi/mmole, was synthetized at AB LEO, Helsingborg, Sweden. The purity, determined by thin layer chromatography, was at least 95%. Carrierfree ^{65}Zn as the chloride, thymidine, [methyl-^{3}H], specific activity 20 Ci/mmole, and estradiol-17β, [2, 4, 6, 7, 16, 17-^{3}H], specific activity 152 Ci/mmole, were purchased from New England Nuclear, 06072 Dreieichenhein, W. Germany. Dihydrotestosterone, [1, 2, 4, 5, 6, 7-^{3}H], specific activity 165 Ci/mmole, and progesterone, [1, 2, 6, 7-^{3}H], specific activity 94 Ci/mmole, were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England. The radiolabeled steroid hormones were purified on Sephadex LH 20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Duplicates of tissue samples (200–400 mg) and blood (0.5 ml) were combusted in a Packard Tri Carb Sample Oxidizer Model 306 and were analyzed for tritium activity in a Packard Tri Carb Liquid Scintillation Spectrometer Model 2650 or in a Philips PW 4510-00 Liquid Scintillation Analyzer for at least 10 min or 4×10^5 dis/min. Plasma and extracts from tissues and thin layer chromatograms were analyzed directly. Quenching was corrected by the external standard ratio method. Samples containing ^{65}Zn were analyzed in a Packard Autogamma Scintillation Spectrometer Model 5210.

Thin layer chromatography (TLC) was performed on tissue extracts obtained by homogenizing tissue samples in 2×3 vol of cold methanol and subsequent centrifugation, and on ether extracts of plasma (2×2 vol). The extracts were evaporated to a volume suitable for spot application on heat-activated, precoated TLC plates (silica gel F_{254}, thickness 0.2 mm) (Merck AG, Darmstadt, W. Germany). The plates were run in a chloroform/ethyl acetate (4:1, v/v) system. Spots of reference compounds were visualized by u.v. light. After extraction of 1×1 cm pieces of the chromatograms for 30 min in 1 ml of methanol the radioactivity content was analyzed.

Statistical evaluation of the results were performed by methods described by Armitage [16].

Tumor growth inhibition

For study of the effects of LS 1727 on tumor growth 2 experiments were performed. In the first experiment 40 tumor-bearing animals were divided into 4 experimental groups approximately equivalent with regard to number and size of the tumors. LS 1727, suspended in a vehicle consisting of carboxymethylcellulose (0.6%), Tween 80 (0.2%) and glucose (5.5%) in water, was injected intraperitoneally once daily for 4 weeks in the doses 10, 20 and 40 mg/kg/day. The animals in the control group were injected with the vehicle. In the second experiment 3 groups of 10 animals were injected with 0.31 or 1.25 mg/kg of LS 1727 or with the vehicle. The size of the tumors was measured twice weekly during the treatment period. The tumor diameters were registered also during the 4 weeks following the treatment period. The appearance and growth of additional tumors was observed throughout the experiment. The body weight was registered once weekly.

Comparative tumor growth inhibition

Tumor-bearing animals were divided at random into 4 experimental groups with 6 animals per group. The animals were injected intraperitoneally once daily for 4 weeks with suspensions of LS 1727, 3.5 mg/kg, 19-nortestosterone, 2.35 mg/kg, CCNU, 2.0 mg/kg, or with the suspending vehicle, and were sacrificed on the day after the last injection. The tumor size and number was registered twice weekly. Twenty-four hours prior to sacrifice 3 animals in each group were injected intraperitoneally with 25 μCi of ^{65}Zn. One hour prior to sacrifice the remaining 3 animals in each group were injected with 100 μCi of ^{3}H-thymidine. At termination tumors, uteri, ovaries and adrenals were dissected and weighed. From each animal injected with

^{65}Zn samples of 2 tumors were taken for analysis of radioactivity concentration. From each animal injected with ^{3}H-thymidine samples of 2 tumors were taken for analysis of concentration of protein [17], DNA and RNA [18, 19] as well as for analysis of ^{3}H incorporation into DNA [20]. Samples from 1 tumor per animal were taken for histological examination and for histochemical investigation of activity and distribution of acid and alkaline phosphatase, nonspecific esterases, leucine aminopeptidase, and β-glucuronidase [21].

polyethylene glycol 400 (0.5 ml/kg). The animals in the 3 groups were sacrificed by exsanguination via the aorta 0.5, 4 and 24 hr, respectively, after injection. The radioactivity concentration in tumor tissue and other tissues was determined. Thin layer chromatography was performed on extracts of tumor, liver, kidney and plasma.

RESULTS

Tumor growth inhibition

Table 1 shows that LS 1727 caused a dose-dependant reduction of tumor growth. At the end of the 4 week treatment period even the lowest dose, 0.31 mg/kg, had a statistically significant effect. Tumor growth was not restored during the 4 week observation period following the treatment period. Table 2 shows that LS 1727 also caused a dose-dependant reduction of the tumor number increase. LS 1727 caused a negligible mortality during the treatment period (Table 3). However, the highest dose, 40 mg/kg, caused a marked mortality during the observation period and also a reduction of body weight.

Table 1. Growth inhibition of DMBA-induced mammary tumors by LS 1727

Daily dose of LS 1727 mg/kg	Tumor growth week 4		Tumor growth week 8	
	Percentage of growth in control animals*	95% confidence limits	Percentage of growth in control animals	95% confidence limits
40	29	19–45	—	—
20	42	30–60	27	18–40
10	48	30–77	32	21–49
1.25	53	38–75	64	46–89
0.31	65	44–98	77	52–113

*The sum of the average tumor diameters for each animal in the experimental groups was compared to the mean sum of average tumor diameters for the control animals.

Hormone uptake inhibition

Tumor-bearing animals were divided at random into 9 experimental groups with 5 animals per group and were ovariectomized. Twenty-four hours later the animals were injected intraperitoneally with a solution of LS 1727 in 25% ethanol, 1000 μg per animal (3 groups) or 10 μg per animal (3 groups) or with the vehicle (3 groups). Thirty minutes later a solution (10% ethanol in saline) of 5.8 μCi of ^{3}H-dihydrotestosterone, 3.3 μCi of ^{3}H-estradiol-17β, or 4.1 μCi of ^{3}H-progesterone was injected intravenously into the animals, each steroid to one group pretreated with LS 1727, 1000 μg, to one group pretreated with LS 1727, 10 μg, and to one group pretreated with the vehicle. Two hours later the animals were sacrificed and the ^{3}H concentration in blood, muscle (m. rectus abdominis), uterus, and in 2 tumors per animal was determined.

Distribution and metabolism

Tumor-bearing animals were divided at random into 3 experimental groups of 4 animals. The animals were injected intravenously with 3.5 mg/kg of ^{3}H-LS 1727 dissolved in

Comparative tumor growth inhibition

In this experiment the antitumor effect of LS 1727 was compared to the effect of equimolar doses of 19-nortestosterone, the hormonal moiety of LS 1727, and of CCNU, a compound with structural similarity to the alkylating moiety of LS 1727. The alkylating moiety proper can not be isolated owing to its chemical instability. Table 4 shows that tumor growth was significantly retarded by LS 1727 but not by the other compounds. No significant effect on tumor number per animal was obtained by any of the treatments. LS 1727 increased the weight of the uterus and decreased the weight of the ovaries (Table 5). The adrenal weight was

Table 2. Tumor number change in control animals and in animals given LS 1727

Daily dose of LS 1727 mg/kg	Tumor number per animal		
	Week 0	Week 4	Week 8
—	2.6±0.4*	6.7±0.6	8.6±0.9
40	2.7±0.4	3.6±0.7	—
20	2.3±0.3	3.4±0.3	3.4±0.5
10	2.7±0.4	3.6±0.4	3.8±0.5
—	2.6±0.3	9.7±0.7	10.5±1.0
1.25	2.7±0.3	6.4±0.8	8.5±1.1
0.31	2.7±0.3	8.2±1.1	10.3±1.3

*Mean ± standard error.

Table 3. Mortality and body weight change in control animals and in animals given LS 1727

Daily dose of LS 1727 mg/kg	Mortality (dead/total)		Body weight, g		
	Week 4	Week 8	Week 0	Week 4	Week 8
—	0/10	1/10	261±5†	289±6	292±4
40	1/10	8/10	262±3	230±7	
20	0/9	1/9	258±4	251±4	258±6
10	1/10	1/10	259±5	269±6	275±7
—	0/10	2/10	251±4	271±8	285±7
1.25	0/10	0/10	246±5	276±8	280±7
0.31	0/10	0/10	245±3	271±3	284±4

†Mean ± standard error.

Table 4. Effect of equimolar doses of LS 1727, 19-nortestosterone and CCNU on tumor growth and tumor number

Compound	Daily dose mg/kg	Sum of tumor diameters per animal, mm		Number of tumors per animal	
		Day 0	Day 28	Day 0	Day 28
Vehicle	—	25±5‡	153±30	2.6±0.7	10.4±2.5
LS 1727	3.5	25±3	70±17	2.5±0.5	5.8±0.7
19-nortestosterone	2.4	33±4	116±12	3.5±0.4	7.2±0.7
CCNU	2.0	24±1	99±20	2.0±0.3	9.3±2.0

‡Mean ± standard error.

Table 5. Organ weights and body weights in animals given equimolar doses of LS 1727, 19-nortestosterone, and CCNU

Compound	Daily dose mg/kg	Uterus, mg	Ovaries, mg	Adrenals, mg	Body weight, g	
					Day 0	Day 28
Vehicle	—	374±32§	130±14	75±8	251±7	274±9
LS 1727	3.5	697±31	82±5	63±3	248±6	279±11
19-nortestosterone	2.4	493±83	114±4	78±6	245±4	269±12
CCNU	2.0	309±40	102±8	68±9	257±4	247±7

§Mean ± standard error.

Table 6. Effect of equimolar doses of LS 1727, 19-nortestosterone, and CCNU on DNA synthesis, on concentration of DNA, RNA, and protein, and on uptake of ^{65}Zn in DMBA-induced mammary tumors

Compound	Daily dose (mg/kg)	DPM/μg DNA	DNA (μg/mg)	RNA (μg/mg)	Protein (μg/mg)	^{65}Zn permillage of given dose/g
Vehicle	—	119±5*	5.5±0.4	4.3±0.2	133±5	6.0±0.2
LS 1727	3.5	34±10	5.4±0.4	4.1±0.1	120±6	5.1±0.4
19-nortestosterone	2.4	90±20	6.0±0.2	4.8±0.2	128±4	5.4±0.3
CCNU	2.0	62±22	5.7±0.7	4.3±0.2	127±5	7.4±0.3

*Mean ± standard error.

Table 7. Effect of LS 1727 on radioactivity concentration, permillage of given dose/g wet weight or ml, *in tumor tissue and other tissues after administration of ^{3}H-dihydrotestosterone, ^{3}H-estradiol-17β, or ^{3}H-progesterone*

Radiolabeled steroid	Pretreatment	Tumor	Uterus	Muscle	Blood
Dihydrotestosterone	Vehicle	0.40±0.03†	0.22±0.03	0.18±0.02	0.32±0.04
Dihydrotestosterone	LS 1727, 10 μg	0.26±0.03	0.16±0.03	0.14±0.02	0.23±0.03
Dihydrotestosterone	LS 1727, 1000 μg	0.17±0.03	0.16±0.05	0.14±0.04	0.24±0.06
Estradiol-17β	Vehicle	8.74±0.53	40.3±6.3	1.29±0.03	0.86±0.03
Estradiol-17β	LS 1727, 10 μg	9.43±1.03	39.4±5.0	1.25±0.05	0.78±0.06
Estradiol-17β	LS 1727, 1000 μg	6.97±0.66	16.0±3.7	0.74±0.11	0.66±0.05
Progesterone	Vehicle	0.51±0.04	0.37±0.03	0.41±0.07	0.52±0.07
Progesterone	LS 1727, 10 μg	0.58±0.07	0.43±0.07	0.40±0.06	0.67±0.11
Progesterone	LS 1727, 1000 μg	0.51±0.06	0.44±0.07	0.42±0.08	0.57±0.08

†Mean ± standard error.

not affected by any of the compounds. The body weight in animals given CCNU was lower than the body weight in the control animals. The incorporation rate of ^{3}H-thymidine in tumor DNA was reduced to 30% by LS 1727 ($P<0.001$) and to 50% by CCNU ($P<0.05$) (Table 6). The concentration of DNA, RNA and protein was not significantly affected. The uptake of ^{65}Zn in the tumors was increased to 120% ($P<0.001$) by CCNU. The histological and histochemical examinations did not reveal any obvious difference between tumors from control animals and tumors from animals given LS 1727, 19-nortestosterone or CCNU.

Hormone uptake inhibition

The ^{3}H concentration in tumor tissue after injection of ^{3}H-dihydrotestosterone was significantly reduced by pretreatment with LS 1727 (Table 7). The isotope concentration in muscle, uterus and blood was not affected. In the parallel experiment where ^{3}H-estradiol-17β was injected the low dose of LS 1727 did not affect the high uptake of ^{3}H-labeled material in the tumors or the uterus (Table 7). The high dose, however, significantly ($P<0.05$) reduced the uptake in the uterus and in blood and muscle ($P<0.01$) but not the uptake in the tumors. The concentration of ^{3}H-labeled material in tumor, uterus, muscle and blood after injection of ^{3}H-progesterone was not affected by LS 1727 (Table 7).

Distribution and metabolism

The distribution kinetics in terms of tissue specific activity after administration of ^{3}H-LS 1727 are shown in Fig. 2. At all sampling times the concentration of radioactivity in liver and kidney was significantly higher ($P<0.05$) than the concentration in blood and other organs and tissues. The concentration in the tumors tended to be higher than the concentration in muscle. Radioactivity concentration in all organs declined with time approximately parallel with the decline in blood concentration. This indicates that no accumulation of radioactivity occurred in any of the organs examined.

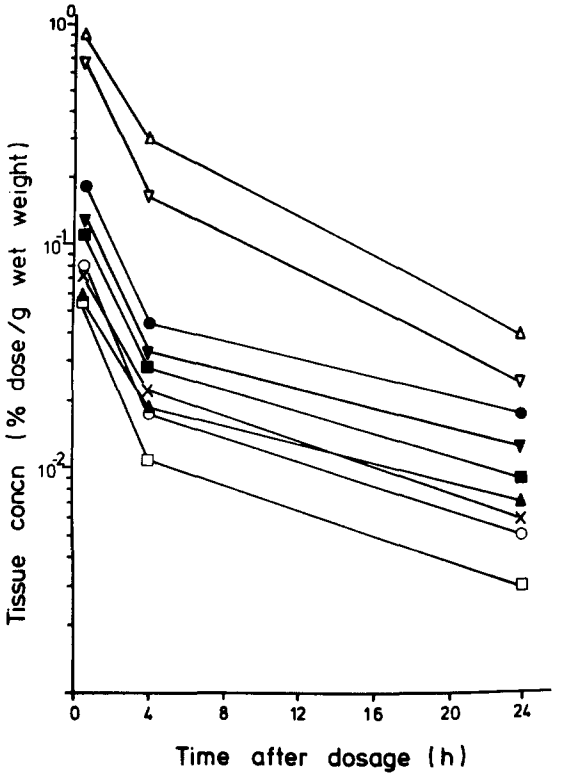

Fig. 2. Tissue concentrations of radioactivity, following a single intravenous dose of ^{3}H-*LS* 1727 (3.5 *mg/kg*). *Standard errors were less than* 25% *of the means* (n=4). ▼, *blood;* ●, *plasma;* △, *liver;* ▽, *kidney;* ■, *lungs;* □, *muscle;* ▲, *spleen;* ×, *uterus;* ○, *tumors.*

Thin layer chromatography revealed that only negligible amounts of unchanged LS 1727 were present in the tissues examined. The principal extractable metabolite in plasma 30 min after injection had the same R_F-value as 19-nortestosterone and less than 5% of the radioactivity could be accounted for as LS 1727. Most of the radioactivity extracted from plasma samples obtained 4 or 24 hr after injection remained at the application spot of the chromatogram which indicates high levels of polar metabolites.

Essentially all the radioactivity found in the liver and kidney 0.5, 4 or 24 hr after injection could be accounted for as polar metabolites. The metabolite pattern in the tumors were similar to those in the liver and kidney except that the tumors 30 min after injection contained relatively high amounts of a metabolite with the same R_F-value as 19-nortestosterone besides polar metabolites.

DISCUSSION

The growth of DMBA-induced mammary tumors in rats is affected by various hormones and cytostatic agents [8, 9, 11, 12]. It has recently been shown that also two hormone-linked cytostatic agents can affect tumor growth [10, 22]. The effect of these two compounds was found to be superior to the effect of the hormonal and/or cytostatic moieties of the compounds. In the present report we present results from antitumor activity studies of a third hormone-linked cytostatic agent coded LS 1727, a nitrosocarbamate of 19-nortestosterone.

The results clearly show that LS 1727 has a dose-dependant growth-inhibitory effect on DMBA-induced mammary tumors. The effect persists long after cessation of treatment. The tumor number increase was reduced by LS 1727. The antitumor effects were obtained to the price of only modest toxicity. In a comparative study LS 1727 proved to have a higher effect than equimolar doses of its hormonal moiety, 19-nortestosterone, or of CCNU, a compound with structural similarity to the alkylating moiety in LS 1727.

The action of LS 1727 on the tumors may at least partly be a consequence of a cytostatic action of the alkylating part of the molecule. This is supported by results from our laboratories showing that LS 1727 has a cytostatic action on various non-hormonally dependant experimental tumors [15]. Furthermore, nitrosoureas including CCNU are known to retard growth of DMBA-induced mammary tumors [11]. However, it seems likely that an alkylating action of LS 1727 on the DMBA-induced tumors is supplemented by a hormonal action of the 19-nortestosterone part of the molecule. Several studies have demonstrated the effectiveness of substituted and unsubstituted androgens against DMBA-induced mammary tumors [8, 11, 12], and certain 19-nortestosterone derivatives are among the more effective agents [11]. An androgenic action of LS 1727 or its metabolites is indicated in the present study by the ability of the compound to diminish the uptake of ^{3}H-dihydrotestosterone and its metabolites in the tumors. In a pilot experiment these tumors were homogenized and cytosol was prepared. The preliminary results show that a considerable percentage of the ^{3}H-activity in the tumors was found in the cytosol where the greater part of the radiolabeled material was bound with high affinity to macromolecules. The amount of radioactivity bound with high affinity was reduced by the pretreatment with LS 1727.

The growth of DMBA-induced mammary tumors is affected not only by androgens but also by estrogens [8, 9]. Therefore it is of interest that LS 1727 appears to have an

estrogenic effect besides that androgenic one. Our results show that long-term treatment of female rats with LS 1727 results in an increase of uterine weight. Such a weight increase is usually indicative of an estrogenic action [23]. It is noteworthy that 19-nortestosterone in an equimolar dose did not cause any weight increase of the uterus.

Corroborative evidence for an estrogenic effect of LS 1727 is given by the results from the competition studies with ^{3}H-estradiol-17β. These results show that LS 1727 diminished the uptake of ^{3}H-estradiol-17β and its metabolites in the uterus. However, the uptake of ^{3}H-labeled material in the tumors was not significantly affected. The reason for the disparate effects of LS 1727 on the estrogen uptake in the uterus and in the tumors remains to be clarified.

The tumor growth retardation by an estradiol mustard was paralleled by an uptake of the compound in the tumors [10]. For LS 1727 there does not seem to be such an uptake in the tumors of at least the steroid part of the molecule. This does not rule out the possibility that LS 1727 could have a direct effect on the tumors. However, such an effect must be a rapid one as the metabolic studies showed that there is a fast formation of 19-nortestosterone from LS 1727. The degradation of the compound underlines the desirability to perform distribution studies also with LS 1727 labeled in other parts of the molecule than the steroid part.

The results of this study show that LS 1727 has a growth-inhibitory effect, probably exerted by both an alkylating and a hormonal action of the compound, on DMBA-induced mammary tumors. Such tumors are considered a suitable experimental model for screening new drugs potentially active in the treatment of breast cancer [9, 11]. Therefore our results suggest that a clinical testing of LS 1727 against breast cancer may be warranted.

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