The effects of ivermectin used in combination with other known anti-parasitic drugs on adult *Onchocerca gutturosa* and *O. volvulus in vitro*

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Abstract

The effects of ivermectin at a concentration of $3{\cdot}13\times10^{-6}$M used in combination with other anti-parasitic drugs on the viability of adult *Onchocerca in vitro* were assessed using MTT colorimetry and worm motility levels. When ivermectin was used against male *O. gutturosa* over a 7 d period in combination with suramin (5×10^{-5}M), CGP 6140 ($3{\cdot}13\times10^{-6}$M), CGP 20376 ($1{\cdot}95\times10^{-7}$M), mefloquine ($3{\cdot}13\times10^{-6}$M), levamisole ($3{\cdot}13\times10^{-6}$M), mebendazole (5×10^{-5}M), flubendazole (5×10^{-5}M) and albendazole (5×10^{-5}M), there was either no increased effect or only a marginally increased effect on motility levels when compared with the use of ivermectin alone. MTT colorimetry revealed that in most cases there was a cumulative effect of the 2 drugs used in combination but not a synergistic effect. In a trial extended to 26 d it was demonstrated that the combination of ivermectin and suramin did not produce a greater inhibition of motility than ivermectin alone. Using female *O. volvulus*, the activity of ivermectin, CGP 6140 and the 2 drugs combined was examined. The motility of all 3 groups exposed to drug(s) was suppressed by 24 h compared with controls. MTT colorimetry performed on day 7, using the pre-weighed anterior end of each worm, illustrated that ivermectin alone produced a 43·4% inhibition of formazan formation compared with controls, CGP 6140 alone produced 50·6% inhibition, while the drug combination produced a 72% inhibition, equivalent to the heat-killed control. The cumulative effects seen with certain drug combinations *in vitro* should be examined *in vivo*, where they may have some practical value in the elimination of adult *Onchocerca*.

Introduction

Ivermectin is an extremely effective single dose *Onchocerca* microfilaricide which greatly reduces parasite levels in the dermis and suppresses the release of new microfilariae from female worms for over 12 months (TAYLOR & GREEN, 1989), while lacking any obvious macrofilaricidal effects. Widespread use of ivermectin (Mectizan®; Merck Sharp & Dohme) is now planned in order to reduce symptoms of disease and possibly to help control transmission (ANONYMOUS, 1989; BRADSHAW, 1989; CUPP *et al.*, 1989). However, ivermectin does not 'cure' onchocerciasis by killing the adult worms, so that repeated dosing at 12 monthly intervals is necessary (TAYLOR, 1987). The primary objective of the onchocerciasis chemotherapy project (WHO, 1985) is to develop an effective drug to eliminate the adult worms, thereby affecting radical cure.

In earlier studies we have demonstrated that ivermectin inhibits motility or paralyses adult male *O. gutturosa in vitro* in a dose-dependent fashion (TOWNSON *et al.*, 1987), but has no lethal effect at concentrations similar to peak plasma levels found in man (BENNETT *et al.*, 1988) following treatment with a 200 μg/kg dose. In the present experiments we have examined the effects of ivermectin used in combination with other known filaricides against adult *O. gutturosa* and *O. volvulus in vitro*, to see if possible cumulative or synergistic effects between drugs may result in significantly increased efficacy.

Materials and Methods

Recovery, isolation and maintenance of parasites

Similar techniques have been described previously (TOWNSON *et al.*, 1989). Briefly, male *O. gutturosa* were isolated by dissection from the nuchal ligament connective tissues of naturally infected cattle following slaughter at a UK abattoir. *O. volvulus* nodules were obtained from patients in Guatemala, and the worms isolated by the digestion of host tissues in collagenase enzyme (type 1A; Sigma, UK) at a concentration of 5 mg/ml at 30°C for periods of 7–20 h (see TOWNSON *et al.*, 1986, based on SCHULZ-KEY *et al.*, 1977). Worms were washed and then cryopreserved at −79°C and air-freighted to the UK on solid CO_2 (see TOWNSON *et al.*, 1989, for freezing and thawing procedures). Following 44–53 d storage at −79°C, female *O. volvulus* were thawed and maintained individually in culture for 24 h in 6 well plates (Falcon) over a monkey kidney cell (LLCMK2) feeder layer. The smaller male *O. gutturosa* were maintained in a similar way in 24-well plates (see TOWNSON *et al.*, 1989 for details) for 24 h before use.

Drug preparation

The ivermectin used was the commercial preparation Ivomec® in liquid form which was diluted into the test medium [Eagle's minimum essential medium (MEM)+10% heat-inactivated foetal calf serum+100 iu penicillin+100 μg/ml streptomycin+0·25 μg/ml amphotericin B+sodium bicarbonate (2 g/litre)]. The remaining drugs were prepared as described previously (TOWNSON *et al.*, 1987), or first dissolved in dimethylsulphoxide (DMSO) before addition to the test medium. When DMSO was used to prepare the drug, then an equivalent quantity (not exceeding 0·016%) was added to control wells. Drug concentrations were chosen which had previously been shown partially to inhibit worm motility (TOWNSON *et al.*, 1987), so that any possible synergistic effects could be visualized.

The experiments were set up by adding a male *O.*

gutturosa to each well of a 24-well plate containing 1·8 ml of medium (±drug) over LLCMK2 feeder cells, or by adding a female *O. volvulus* to each well of a 6-well plate containing 8 ml of medium (±drug), also over LLCMK2 cells. Cultures were maintained in an atmosphere of 5% CO_2 in air. In experiment 1, using male worms, the medium/drug was not renewed during the 7 d trial. In experiment 2, examining the long-term effects of suramin and suramin/ivermectin in combination for 25 d, medium/drug were renewed on days 7, 14 and 21. In experiment 3, female *O. volvulus* had medium/drug renewed on days 3 and 6.

Evaluation of drug effects

(i) *Motility.* Motility levels of male *O. gutturosa* were noted every 30 min for the first 4·5 h, then at 1, 2, 3, 4 and 7 d. Motility was scored on a scale of 0 (non-motile) to 10 (maximum normal control activity under optimum conditions) (see TOWNSON *et al.*, 1987). Motility levels of the less active female worms were scored from −(non-motile) to +++(maximum motility), consisting of coiling and probing of the anterior end with occasional contractions of other parts of the body. Immediately before the experiment was set up, all the worms scored ++ or +++ and were randomly allotted to the different treatment groups.

(ii) *MTT/formazan colorimetry.* We have previously described this assay (COMLEY *et al.*, 1989a, 1989b) for the measurement of worm viability. Briefly, single intact male worms were placed in 0·5 ml of medium containing 0·5 ml mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) and incubated at 36·5°C (±0·5°) for 30 min. At the end of the MTT incubations, worms were removed and carefully transferred to a separate well of a microtitre plate containing 200 µl of DMSO and allowed to stand at room temperature for 1 h to solubilize. Following gentle agitation to disperse the colour evenly, the absorbance (optical density=OD) of the resulting formazan solution was determined at 490 nm in a multi-well scanning spectrophotometer relative to a DMSO blank. For female *O. volvulus*, the anterior 4 cm of worm were removed, weighed and processed in a similar manner, with results expressed on the basis of mean OD per mg of worm and as the inhibition of formazan formation compared to controls.

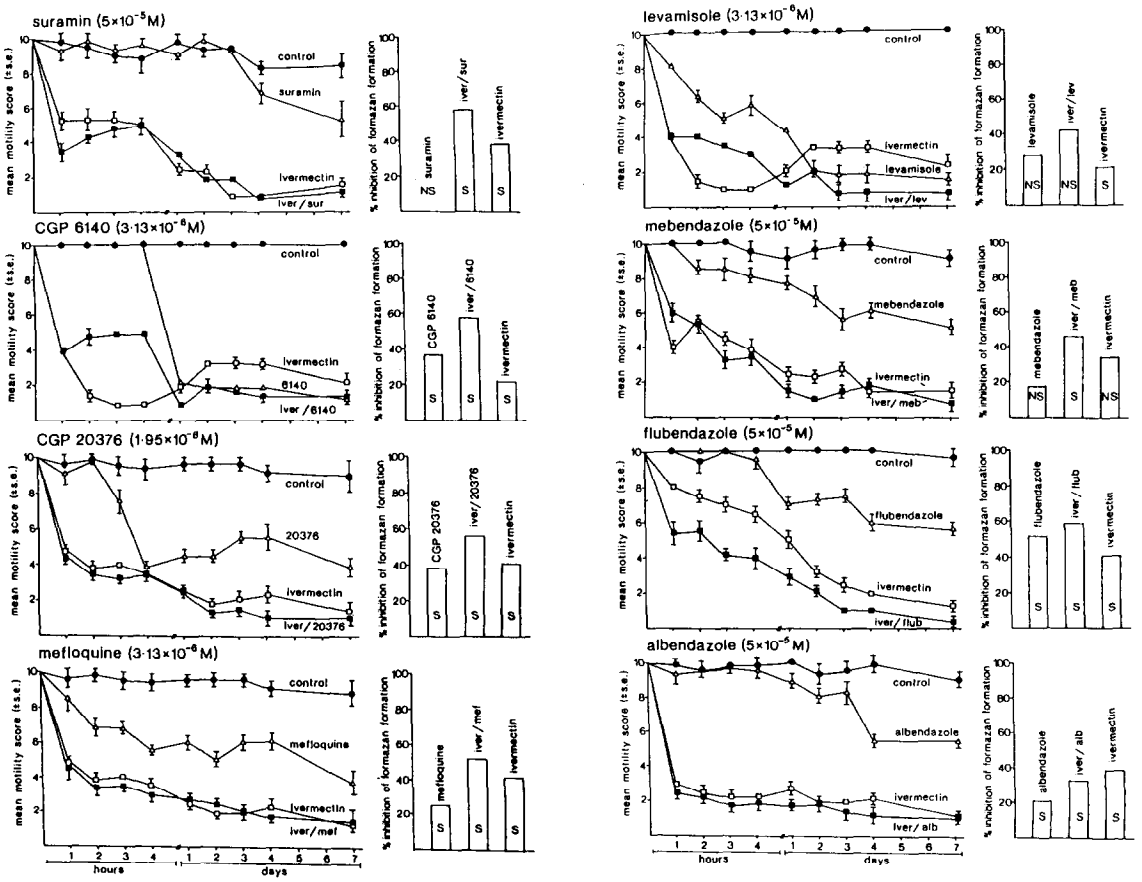

Fig. 1. The effects of ivermectin ($3·13\times10^{-6}$ M) and other antiparasitic drugs, used alone and in combination, on the viability of male *O. gutturosa in vitro* assessed by motility levels and MTT colorimetry. S=significant inhibition ($P<0·05$) of formazan formation compared to controls, NS=not significant. Other comparisons are made in the text.

Results

The activity against male O. gutturosa *of ivermectin used in combination with mebendazole, flubendazole, albendazole, levamisole, suramin, mefloquine, CGP 6140 and CGP 20376*

The effect on male *O. gutturosa* of ivermectin alone, or in combination with a second drug, was examined by means of motility assessments and by the MTT/formazan assay (Fig. 1). Each treatment group consisted of 4–6 worms. In all the experiments the control groups of worms maintained consistently high motility scores (mean 8·3–10 at each time point), with similarly good viability readings from the MTT/formazan formation assay (mean OD readings 0·182–0·258 at 490 nm). In each trial the group of worms exposed to ivermectin alone showed greatly reduced motility levels, following a similar pattern over the 7 d period, resulting in an approximately 80% reduction compared to controls. Each known antiparasitic drug also significantly inhibited worm motility levels ($P<0·05$, two-sample t test on day 7) without completely immobilizing all the worms. However, when ivermectin was used in combination with each drug, there appeared to be little or no effect on motility scores compared to the use of ivermectin alone. Ivermectin alone produced between 22% and 42% inhibition of formazan formation, a statistically significant reduction in most cases (see Fig. 1). With the exception of suramin, all the other drugs produced some inhibition of formazan formation, with significant reductions produced by flubendazole, albendazole, mefloquine CGP 6140 (Ciba-Geigy) and CGP 20376 (Ciba-Geigy), but not by suramin, levamisole and mebendazole. With the exception of suramin, all of these results were close to one side or the other of statistical significance ($P=0·05$).

The drug combinations, with the exception of ivermectin/albendazole, all produced a greater inhibition of formazan formation than each drug alone. The ivermectin/mebendazole combination produced a significant increase in formazan inhibition ($P=0·049$) when compared with mebendazole, but not when compared with the ivermectin group. Similar statistics were obtained in the albendazole experiment, but in the flubendazole experiment there were no significant differences between the 3 groups, although all 3 were significantly different from controls ($P<0·01$). The ivermectin/CGP 6140 combination produced a significantly greater inhibition ($P<0·02$) than either drug alone, while the ivermectin/CGP 20376 combination was significantly more inhibitory compared to CGP 20376 ($P=0·02$) but not when compared to ivermectin alone. The effects of the drug combinations ivermectin/levamisole and ivermectin/mefloquine were not significantly different compared to each drug alone. Finally, the effect of the ivermectin/suramin combination was highly significant ($P<0·005$) when compared to suramin alone (formazan formation values similar to controls), but not when compared to ivermectin alone.

The activity of ivermectin used in combination with suramin over a 26 d period against male O. gutturosa

All worms survived the 26 d trial, with the control group maintaining a mean motility score of 7·3–9·8 throughout (Fig. 2). The ivermectin and ivermectin/suramin groups were rapidly and similarly affected up to the end of the trial, with the pattern of motility scores resembling that seen in the previous experiment over the first 7 d. The worms exposed to suramin alone appeared to be slightly more active than the controls up to 24 h, although there were no significant differences between them up to day 11. However by day 12 the suramin group had declined in motility to a mean of 5 compared to the control value of 8·3, and remained low until the end of the trial on day 26. At this time the suramin group scored a mean of 2 compared with that of controls, 9·8 ($P=<0·01$). Using motility as a measure of viability there appeared to be no synergistic or cumulative effects between those 2 drugs.

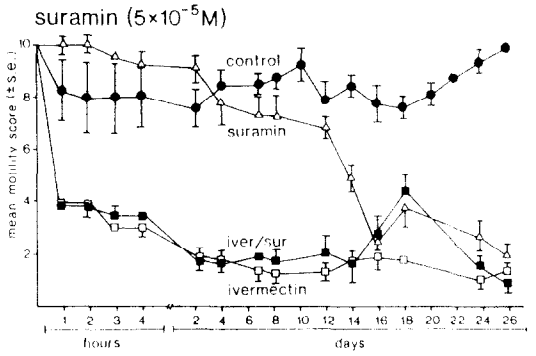

Fig. 2. The long-term effect of ivermectin ($3·13\times10^{-6}$ M) and suramin (5×10^{-5} M), used alone and in combination (iver/sur), on the motility levels of male *O. gutturosa in vitro*.

The activity of ivermectin used in combination with CGP 6140 against O. volvulus *females*

Table 1 illustrates the motility pattern exhibited by individual worms exposed to ivermectin, CGP 6140 and ivermectin/CGP 6140 combined. Despite the fact that the movements of these parasites were somewhat erratic, it was clear that by 24 h the groups of worms exposed to drugs were less active (some non-motile) than controls. On day 3 one control worm became non-motile, while the remaining worms remained active until the end of the trial. The 3 groups of worms exposed to drug remained either non-motile or only slightly active from day 2 onwards. Due to a minor level of bacterial contamination on day 6 in the group of worms exposed to CGP 6140, these worms were assessed for viability using MTT colorimetry one day early.

Formazan formation values (OD/mg) were quite variable between individual worms (Table 2), although a clear pattern emerged with controls scoring a mean of 0·168, the ivermectin group 0·095, CGP 6140 group 0·083 and the combination of both drugs 0·047 (inhibition of formazan formation=72%, $P=0·07$), which was an equivalent value to that of the heat-killed (HK) control. The most damaged worms, therefore, were those exposed to the drug combination.

Discussion

The rather unsatisfactory nature of the drugs available for the treatment of onchocerciasis has focussed attention on the possibility of using 2 drugs in combination with the hope of increasing macrofilar-

Table 1. Motility levels of individual female *O. volvulus* incubated in ivermectin ($3{\cdot}13\times10^{-6}$ M) and CGP 6140 ($3{\cdot}13\times10^{-6}$ M) used alone and in combination[a]

A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
		1 h				1·5 h				2 h				2·5 h	
+++	+++	+	+	+++	+++	++	++	+++	+++	++	++	+++	+++	++	+
+++	++	++	+	+++	++	++	+	+++	++	++	+	+++	++	++	++
+	++	++	+++	++	++	++	+++	+	++	+	++	++	++	+	++
++	++	++	+	++	++	++	+	+++	++	++	++	++	++	++	++
++				++				++				++			
+++				++				++				++			
+				++				b							
		3 h				24 h				48 h				72 h	
+++	++	++	++	+++	++	−	−	+	−	−	−	+	−	−	−
+++	++	++	+	+++	+	+	+	+++	+	+	−	+	+	+	−
++	++	+	++	++	−	−	++	++	+	−	−	−	−	−	−
++	++	++	++	+++	−	++	+	+++	−	+	+	+++	−	+	−
++				++				++				++			
++				+++				++				+++			
		96 h				120 h				144 h				168 h	
+	+	−	−	+	−	−	−	+	+	−	−	+	+	−	ND[c]
+++	+	+	−	+++	+	−	−	+++	−	−	−	+++	+	−	ND
−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	ND
+++	−	+	−	+++	−	+	+	+++	−	+	−	+++	−	+	ND
++				++				+				++			
+++				+++				++				+++			

[a]Vertical columns indicate treatment (A=control, B=ivermectin, C=ivermectin+CGP 6140, D=CGP 6140).
[b]Worm removed for other purposes.
[c]Not done.

icidal effects. The advent of ivermectin, a remarkably effective single dose microfilaricide (AWADZI *et al.*, 1986; BROWN & NEU, 1989), which has non-lethal effects against adult *Onchocerca in vitro* (TOWNSON *et al.*, 1987) and against intra-uterine stages *in vivo* (DIALLO *et al.*, 1986), prompted us to examine its effects in combination with other known filaricides. These experiments demonstrated that, *in vitro*, true synergism did not occur between ivermectin when combined with any of the other drugs examined, i.e. they did not interact to produce an effect greater than the sum of their individual effects. In the trials using male *O. gutturosa*, the motility scores generally indicated only a slight increase in inhibition when the drug combinations were compared to the effects of ivermectin alone. There were some minor differences between individual MTT experiments: for example, ivermectin alone produced between 22% and 42% inhibition of formazan formation. This variability was probably due to small differences in worm size (too small to weigh accurately) and the relatively small number (4–6) in each group. Minute inaccuracies in the concentration of this potent drug may also account for small differences in motility patterns and the level of formazan formation. Nevertheless, the ivermectin effects were generally consistent throughout the series of experiments, with each MTT result (with the exception of ivermectin/albendazole) illustrating a cumulative effect of 2 drugs in combination, i.e. producing an inhibition of formazan formation rather less than, or approximately equivalent to, the sum of their individual effects. None of the drug combinations produced an inhibition of formazan formation equivalent to that recorded (89–97%) with heat-killed male *O. gutturosa* in earlier studies (TOWNSON *et al.*, 1989; COMLEY *et al.*, 1989a).

The longer-term effect of suramin against male *O. gutturosa in vitro* confirmed our earlier observations (TOWNSON *et al.*, 1987) that this drug has only a very limited effect on motility levels during our standard 7 d *in vitro* test. However, by day 14 there was a significant inhibition (37%) of motility, rising to a maximum of 80% inhibition by the end of the experiment (day 26). HOWELLS *et al.* (1983) demonstrated that, *in vivo*, suramin killed *Brugia pahangi* by acting at the surface of the intestinal epithelium, but that the drug was ineffective and unable to penetrate *B. pahangi in vitro* during incubations of 1–72 h, also indicating that the worms were not taking up material orally. Our study suggests that the drug does enter *O. gutturosa in vitro*, but that there is a delayed effect so that long-term cultures are required to visualize activity; this agrees with studies showing a slow lethal effect (up to 7 weeks) against *Brugia in vivo* (DENHAM, 1979; HOWELLS *et al.*, 1983), while the long period required to kill *O. volvulus* in man has been well documented (DUKE, 1968; SCHULZ-KEY *et al.*, 1985). This suggests that, for some novel compounds, greatly extended *in vitro* assays may be required to detect activity.

We have previously demonstrated that the simple technique involving the freezing of female *O. volvulus* over dry ice (−79°C) can be used to transport and store specimens, which, when thawed, may retain motility for up to 272 d in culture (TOWNSON *et al.*, 1989), and which produce good levels of formazan when incubated in MTT (COMLEY *et al.*, 1989a). This study shows that cryopreserved female *O. volvulus* can be used to examine drug effects *in vitro*. It is encouraging that the experiment examining the effects of CGP 6140, which has shown activity against *O. gibsoni* in cattle and *O. volvulus* in clinical trials (STREIBEL, 1986; WHO, 1988), in combination with ivermectin against female *O. volvulus*, produced results very similar to those obtained using the model system employing male *O. gutturosa*. However, the

Table 2. Viability of individual *O. volvulus* females by MTT colorimetry following 7 day incubations in ivermectin ($3{\cdot}13\times10^{-6}$ M), CGP 6140 ($3{\cdot}13\times10^{-6}$ M), and the two drugs combined

Group	Worm no.	Sample weight (mg)	Optical density/mg (490 nm)	Mean optical density	Inhibition of formazan formation compared to control	*P*
Heat-killed control	1	0·7	0·047	0·047	72·0%	–
Control	1	1·6	0·390	0·168±0·11[a]	–	–
	2	1·3	0·138			
	3	1·9	0·060			
	4	1·4	0·153			
	5	1·7	0·166			
	6	1·3	0·103			
Ivermectin	1	2·3	0·169	0·095±0·06[a]	43·4%	0·28
	2	2·3	0·108			
	3	1·8	0·036			
	4	1·3	0·068			
Ivermectin plus CGP 6140	1	1·8	0·051	0·047±0·01[a]	72·0%	0·07
	2	1·3	0·035			
	3	2·2	0·065			
	4	1·9	0·037			
CGP 6140	1	1·9	0·029	0·083±0·06[a]	50·6%	0·22
	2	1·5	0·087			
	3	0·9	0·166			
	4	2·6	0·051			

[a]Standard deviation.

erratic and comparatively low activity of female worms limit the usefulness of motility as a measure of drug activity, although drug effects can be clearly observed by 24 h. The use of the pre-weighed anterior 4 cm of the females in the MTT assay does appear to provide a meaningful measure of drug activity, with the drug combination producing 72% inhibition of formazan formation. However, the formazan formation values were quite variable between worms, with one of 6 control worms apparently dying, probably due to overexposure to collagenase enzyme. The decision to use the anterior 4 cm was based on our earlier observation that this is metabolically the most active part of the worm (COMLEY *et al.*, 1989a), with further work indicating that this piece may be used as an accurate reflection of the overall level of viability (COMLEY *et al.*, 1989b). Clearly it would be preferable to use larger groups of female worms, because of their heterogeneity due to differences in age, reproductive status and condition following isolation with collagenase enzyme, but supplies of viable adult *O. volvulus* are likely to be very limited.

To date, the use of drug combinations has not been particularly encouraging for the treatment of filarial infections. For example, levamisole, a broad spectrum anthelmintic and known immunomodulator (GOLDSTEIN, 1978; ASQUITH, 1980), has been used without much success in combination with mebendazole and flubendazole against *O. volvulus* (AWADZI *et al.*, 1982; DOMINGUEZ-VASQUEZ & RIVAS-ALCALA, 1985), although MAK & CHAN (1983) reported that a combination of mebendazole and levamisole was more effective in treating patients with *Brugia malayi* than mebendazole alone. The fact that some drugs, such as levamisole, may work in association with host factors highlights the limitations of studies *in vitro*, where there are no immune components or metabolic processing of drug.

In this study, most drug combinations produced a cumulative but not synergistic effect, which may be useful and requires further research. For example, if the host were to be first treated with ivermectin to remove skin microfilariae with minimal side effects (WHO, 1989) and to have some possible (non-lethal) effects on adults, followed by dosing with a potential *Onchocerca* macrofilaricide (e.g. CGP 6140), then improved anti-adult effects may be obtained compared with using the macrofilaricide alone, without producing a Mazzotti reaction. The possibility of host factors playing an important part over and above the intrinsic effects observed *in vitro* must also be considered.

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