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Original Article

Evaluation of Efficacy of Formoterol Fumarate on Simvastatin Induced Myopathy in Rats

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Abstract

Skeletal muscle atrophy remains a clinical problem in numerous pathological conditions. Formoterol fumarate – a β_2-Adrenergic receptor agonist, can induce mitochondrial biogenesis to prevent such atrophy. We used a Wistar rat model of simvastatin-induced myopathy to assess the effect of formoterol fumarate for the prevention of simvastatin induced myopathy. Rats were administered simvastatin (80 mg/kg) daily in the presence or absence of formoterol fumarate (25 μg/kg) or saline for 14 days. Biometric parameters, physical tests, creatine kinase (CK), lactate dehydrogenase (LDH), histology of biceps femoris and MTT assay were studied. In contrast to what was observed with simvastatin after 14 day's treatment, formeterol fumarate treatment reduced CK and LDH levels with improved physical performance ; reduced muscle necrosis and improved cell viability. Formoterol fumarate with simvastatin treatment showed improvement in simvastatin induced myopathy. Formoterol fumarate holds a potential role in preventing simvastatin induced myopathy.

Introduction

Statins, the inhibitors of HMG CoA (3-hydroxy-3-methyl glutaryl coenzyme A) reductase, have revolutionized the management of cardiovascular disorder like hyperlipidemia and atherosclerosis by a 30% decrease in morbidity and mortality (1). Statins inhibit the rate-limiting HMGCoA reductase enzyme in the mevalonate pathway, which blocks de-novo synthesis of cholesterol and thereby promotes low-density lipoprotein cholesterol uptake into cells (2). However, 0.5%-15% of statin recipients develop adverse effects on skeletal muscles, ranging from slight myalgia to rhabdomyolysis, while muscle pain affects up to 30% of statin users (3). The number of patients reporting muscle-related symptoms was highest in those receiving simvastatin (18.2%), followed by atorvastatin (14.9%), pravastatin (10.9%) and fluvastatin (5.1%) (4).

Although no clear molecular basis exists to explain the myopathy induced by statin, one study presented the molecular basis of statin-induced myopathy by showing that simvastatin administration impaired PI3k/Akt signaling and up-regulated FoxO transcription factors and downstream gene targets known to be implicated in proteosomal and lysosomal mediated protein breakdown, muscle carbohydrate oxidation, oxidative stress and inflammation in an *in vivo* model of statin induced myopathy (5). Another study highlighted the importance of Akt/mTOR

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signaling pathway in statin induced myotoxicity revealing potential drug target for treatment of patients with statin-associated myopathy (6).

Currently, the only effective treatment for statin induced myopathy is the discontinuation of statin use in patients affected by elevated CK levels, muscle aches and pain. Many times, physicians may apply an alternate-day dosing, lower starting doses or two times weekly dosing with longer half-life statins (7).

L-Carnitine, creatine and ω3-PUFA supplementation have been tried for beneficial effect in certain myopathies (8). Nonetheless, to date heterogeneous pathophysiology of long-term and short-term effects of supplementation in myopathies is still missing. In fact, the proof of possible harmful effects in short-term settings (creatine in McArdle disease and ω3 PUFA in animal models of dystrophy and cardiomyopathy) and the absence of studies on the possible harmful effects of prolonged supplementations rigorously highlights that thorough studies are needed before these supplements could be suggested as a treatment in selected muscle diseases (8).

Formoterol fumarate is a long acting β_2 agonist used in asthma and COPD (9). Formoterol fumarate is an activator of the protein known as mTOR, which then induces muscle protein synthesis via inhibition of myostatin (10). It is an important signaling pathway involved in the control of skeletal muscle mass through the regulation of translation initiation, and decreases the expression of muscle atrophy F-box (MAFbx)/atrogin-1 and Muscle-RING finger protein (MuRF) 1, two atrophy-related genes (11, 12). Regeneration of skeletal muscle is a crucial action that adds to the maintenance of muscle function and muscle mass during life. Increased protein synthesis and decreased protein degradation has been shown in rat during administration of β_2 agonist ensuing in a net rise in myofibrillar protein content (13, 14).

In context with stated mechanism of formoterol fumarate, the effect of formoterol fumarate on simvastatin induced myopathy in rats was studied. The animals were treated with formoterol fumarate for 14 days in a preventive model administering 80 mg/kg/day of simvastatin for 14 days to induce myopathy in rats in line with study conducted by Westwood et al. (2005) (15). Since simvastatin enters the cell easily due to its lipophilicity nature, it was selected to induce myopathy in the present study (16). Selection of formoterol fumarate dose was based on a study conducted by Joassard et al., (17). Thus primary objective of the study was to investigate efficacy of formoterol fumarate in preventing simvastatin induced myopathy in rats.

Objective

To assess the effect of formoterol fumarate in simvastatin induced myopathy on rats.

Materials and Methods

Animals

Wistar rats weighing 250-300 gm used in the study were maintained on a constant temperature (22±2ºC), constant relative humidity (55±10%) and automatically controlled 12:12 h light dark cycle (light on at 07:00 hrs). They were fed with standard laboratory food and water ad libitum. The animal handling protocols were based on the CPCSEA guidelines. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of L.M. College of Pharmacy, Ahmedabad.

Drugs

Formoterol fumarate (Vasmi Lab, Mumbai) was dissolved in water while simvastatin in 0.5% m/v CMC. Drugs formoterol fumarate (25 μg/kg) and simvastatin (80 mg/kg) were administered orally daily using oral gavages. All other chemicals were of analytical grade procured from local suppliers.

Experimental procedure

The animals were divided into 4 groups consisting of 6 animals in each group. Group I (normal control) animals were given saline. Group II (diseased control) animals were treated with simvastatin (80 mg/kg) alone, Group III (treatment control group) animals were treated with formoterol fumarate alone (25 μg/

kg) while Group IV (treatment group) animals were treated with simvastatin (80 mg/kg) and formoterol fumarate (25 μg/kg) respectively. In all groups, treatment was given orally for 14 days. Biometric and physical activity parameters were performed at 0,7 and 14 days while CK, LDH, histology of biceps femoris, blood pressure, ECG and MTT assay were performed at baseline and end of treatment (14 days).

Evaluation parameters

Biometric parameters: Body Weight, Food and Water Intake

Biometric parameters including body weight, food intake and water intake were noted every week in all groups. The amount of consumed water and food was calculated by subtracting amount of food and water remaining from amount given before 24 hrs.

Physical activity: Grip strength and Locomotion activity

Grip strength of the animals was studied by placing animals on a Rota rod. The fall time was recorded thrice for each animal and average time was considered. Locomotor activity was recorded using a Actophotometer. The animals were placed in an actophotometer for 5 minutes duration and locomotor activity was recorded as counts.

Biochemical Estimations: Creatine kinase (CK) and Lactate Dehydrogenase (LDH)

Serum CK and LDH levels were assayed using commercially available kits from Beacon diagnostics (Navsari, Gujarat, India).

Hemodynamic Parameters: ECG and Blood pressure

ECG was recorded of each animal using standard bipolar limb lead II method using BIOPAC data acquisition system. Blood pressure was measured by non-invasive tail cuff inflation method using NIBP IWORX 304 system.

Histology and Necropsy

Biceps femoris from the left hind limb of each animal was used for histological examination. Tissues were fixed in buffered 10% formalin, processed to wax blocks, and then sectioned and stained with haematoxylin and eosin for examination using light microscopy with 400x magnification.

Cell-line study: MTT Assay

C2C12 cells were seeded in 96 well culture plates at the density of 2×10^4 cell/cm^2, then incubated with saline, 10 μM simvastatin, 10^{-6}M formoterol fumarate treated cells for 48 hrs. The viability of C2C12 cells was measured by MTT assay. For MTT assay the cell culture with MTT solution in microplate were kept for incubation (4 hr) where in due to the metabolic activity of viable cells MTT is converted into a water insoluble formazan salt. By using Eliza plate reader the amount of formazan were quantified at 550-600 nm.

Statistical Analysis

Data are expressed as the Mean±SEM for the number of animals in each group. Statistical analysis was performed using ANOVA followed by Tuckey's multiple t-tests; two-way ANOVA followed by Tuckey's multiple tests, and multiple t-tests. The level of significance was set at $p<0.05$. The statistical analysis of data was performed using Graph pad prism software.

Results

Biometric Parameters: Effects on body weight, food intake and water intake

During and following 14 days treatment period, no significant ($p>0.05$) difference in body weight, food intake and water intake was observed (Table I) in any of the groups.

Physical activity: Effect on Grip strength and Locomotor activity

At 14 days as compared to normal rats, simvastatin treated animals showed significant ($p<0.05$) reduced physical activity as measured by the ability to stay on the rota rod. Treatment with formoterol fumarate of simvastatin treated animals for 14 days showed

TABLE I: Effect of formoterol fumarate and simvastatin treatment on biometric parameters and physical tests.

Parameter		*Days*	*Normal (N=6)*	*Simvastatin treated (n=6)*	*Formeterol fumarate treated (n=6)*	*Simvastatin and Formeterol fumarate treated (n=6)*
Biometric	Body weight (g)	0	253.33±0.401	262.8±0.401	262.6±0.654	261.6±0.557
		7	254.16±1.851	262.5±0.341	263.6±0.760	262.6±0.557
		14	254±0.557	261.6±0.557	264±0.516	262.3±0.557
	Food intake (g)	0	102.2±1.01	111±4.58	121±5.56	121±4.30
		7	100±0.828	120±3.425	120±3.890	135±3.688*
		14	92.5±8.614	103.1±9.338	108.2±9.675	109.6±9.901
	Water intake (ml)	0	252.6±1.49	244.6±2.62	247.6±5.06	215.5±2.68
		7	253.7±1.64	243.7±2.41	249±4.0	215.1±2.29
		14	226.5±21.0	214.4±19.7	228±20.0	192.6±17.7
Physical test	Rotarod test (sec)	0	21±1.79	18±0.37*	17.67±2.9*	16.83±1.47*
		7	20.33±0.80	13±1.33*	18.33±0.88@	14.67±1.12*
		14	20.33±0.61	12.33±1.26*	18.83±0.70@	14.67±1.15*
	Open field test (no. of counts)	0	133.6±6.83	159.1±6.70	120.5±9.99	116±9.67@
		7	131.83±5.79	114.6±10.05*	124.3±10.90	155.6±5.31@
		14	129.1±4.53	109±8.40*	122±7.59@	150.1±3.79

*p<0.05, **p<0.01,***p<0.001and ****p<0.0001 when compared to normal control group; @p<0.05, @@p<0.01 and @@@p<0.001 when compared to disease control group.

improved physical activity as compared to simvastatin treated group however it was not to equal to normal group (Table I).

Biochemical parameters: Creatine kinase (CK) and Lactate Dehydrogenase (LDH)

Serum CK level were significantly (p<0.0001) higher in simvastatin treated group as compared to normal group after 14 days of treatment. Formoterol fumarate treatment did not show significant difference in serum CK level as compared to the normal group. Treatment with formetrol fumarate to simvastatin treated group significantly improved both CK and LDH levels which were closely similar to normal animals (Table II).

Hemodynamic parameters: ECG recording

There was no significant difference in heart rate, QRS amplitude, QT-interval and P amplitude after treatment with formoterol fumarate treatment compared to normal group (Table II).

Non-invasive Blood pressure

There was no significant change in mean arterial, systolic and diastolic blood pressure following any treatment. The blood pressure was similar to normal group (Table II).

TABLE II: Effect of formoterol fumarate and simvastatin treatment on biochemical tests and hemodynamic parameters.

Parameter		*Normal (N=6)*	*Simvastatin treated (n=6)*	*Formeterol fumarate treated (n=6)*	*Simvastatin and Formeterol fumarate treated (n=6)*
Biochemical test	CK Activity (U/L)	317.1±1.75	498.5±2.65*	317.2±4.17@	312.6±6.76@
	LDH Activity (U/L)	618.1±3.32	784.4±6.86*	588.8±15.26@	608.6±6.66@
Hemodynamic parameter	Heart rate (Beats/Min.)	363.1±3.51	363.6±3.23	365±2.76	366.5±2.88
	QRS amplitude (mV)	0.57±0.024	0.57±0.026	0.58±0.027	0.58±0.013
	QT- interval (mili sec.)	38.6±0.88	37.8±1.40	38.6±0.98	40.1±1.01
	P amplitude (mV)	0.045±0.0029	0.046±0.0027	0.047±0.0037	0.045±0.0032
Non invasive blood pressure	Systolic BP (mmHg)	114.8±0.47	115±0.5	115.6±0.421	116.1±0.477
	Mean arterial blood pressure (mm Hg)	88.1±0.600	91±0.1	90.1±0.600	90.3±0.714*
	Diastolic BP (mmHg)	74.8±0.927	77±.1	77.4±1.019	77.4±0.934

*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 when compared to normal control group; @p<0.05, @@p<0.01 and @@@p<0.001 when compared to disease control group.

Histology and Necropsy

Histological analysis of biceps femoris muscle was done using light microscopy with a magnification of 400X. Fig. (A) depicts histological examination of biceps femoris muscle sections of normal rats treated with saline. It reveals normal skeletal muscle structure. Simvastatin administration for 14 days caused degenerative changes in muscles, in the form of, separation of myofibrils, lost cross striations, enlarged edematous muscle fibers and central nucleation (marked as arrows in Fig. B). The muscle necrosis was characterized by cytoplasmic eosinophilia with loss of cytoplasmic structure and vacuolization. Necrosis was more widespread; there was infiltration by mononuclear and polymorph nuclear cells, edema, vacuolization with fragmentation and loss of cytoplasm. Fig. C depicts section of biceps femoris muscle sections of formeterol fumarate treated animals with well developed muscle cells. Fig. D shows histopathology of simvastatin and formeterol fumarate treatment group which indicated a low incidence of necrosis with little or no inflammatory infiltrate very similar to normal muscle section.

MTT assay

The viability of C2C12 cells as measured by MTT assay showed simvastatin treated cells with significant decrease in cell viability as compared to normal treated cells ($p<0.001$). While formoterol

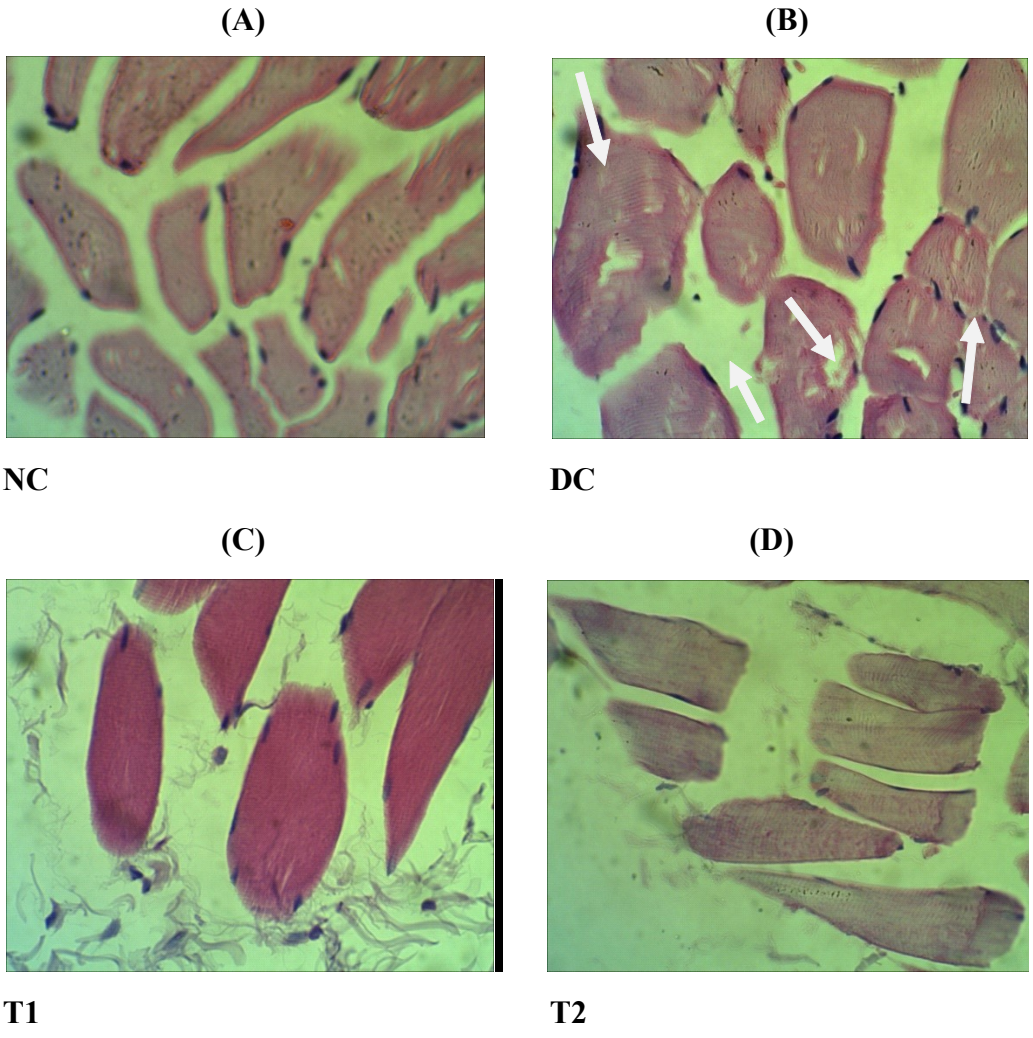

Fig. 1: Histopathology of biceps femoris transverse section hematoxylin and eosin stain.

fumarate alone as well along with simvastatin showed significant increase in cell viability ($p<0.001$) (Fig. 2).

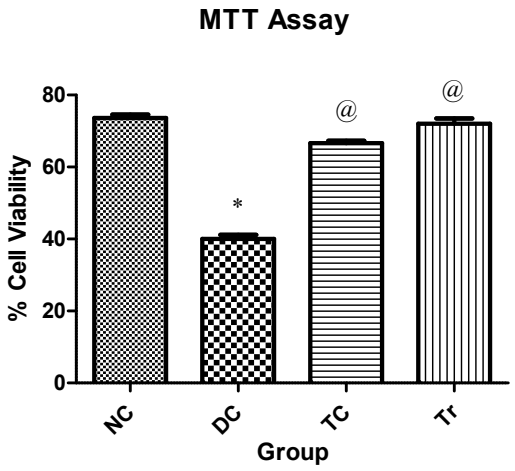

Fig. 2: Effect of formoterol fumarate and simvastatin on % cell viability.
*$p<0.05$, when compared to normal group;
@$p<0.05$ when compared to disease control group.

Discussion

Statins, inhibitors of 3-hydoxy-3-methylglutaryl coenzyme A (HMGCoA) reductase have a beneficial role in prevention of primary and secondary coronary heart disease (2). Therapy of statin appreciably raises the chances of myopathy that can array from muscle aches, weakness and fatigue to fatal rhabdomyolysis (4). Currently, withdrawal of statins is the only available option for statin induced myopathy. Formeterol fumarate is a newer generation beta-2 agonist, an activator of protein mTOR which induces muscle protein synthesis; anabolic actions regulating muscle mass and regeneration. Based on these reviews a preventive model was designed to study the efficacy of formeterol fumarate on simvastatin induced myopathy in rats. This is the first study to demonstrate effect of formoterol fumarate on simvastatin induced myopathy.

Animals were divided in four groups for comparative analysis viz. Normal (Normal Control-saline), simvastatin treated (Disease Control-80 mg/kg orally), formeterol fumarate treated (Treatment Control-25 μg/kg) and simvastatin (80 mg/kg) and formeterol fumarate (25 μg/kg treated (Treatment Group). All treatments were given orally for a period of 14 days.

In line with the observations of Craig and Evelyn, none of the study treatments influenced biometric parameters of body weight, food intake and water intake (18).

Since myopathy is associated with progressive loss of muscle strength, fatigue, myalgia, tenderness, stiffness, cramps, and tightness which ultimately worsens the function of skeletal muscle motor activity (19) damage to skeletal muscle was assessed by physical tests using rota rod and photoactometer at baseline and end of treatment. Motor activity and motor functions were evaluated as the ability of the rodent to maintain balance and keep speed with a rotating rod. These techniques have been used with varying degree of success over years (20). In the present study there was a significant decrease in muscle strength and locomotion in simvastatin treated animals as compared to normal animals ($p<0.0001$). Formoterol fumarate treated animals along with simvastatin administration showed improved muscle strength and locomotion of rats as compared to simvastatin treated animals ($p<0.0001$) suggesting improved physical activity by formetrol fumrate in simvaastatin induced myopathy.

The performance of the skeletal muscle system was also evaluated by testing each rat to straighten itself on four legs when turned on its back alterations of which represent myotonic disorder (21). Righting reflex of animals was also observed which revealed that simvastatin administration scored 2 after completion of treatment (14^{th} day). However formoterol fumarate along with simvastatin administration showed score 0 after 14 days of treatment. This indicates recovery of motor function and coordination after formoterol fumarate administration.

After 14 days of treatment rats were sacrificed, blood was collected and serum was separated for evaluation of biochemical parameters. Biomarkers of skeletal muscle injury like creatine kinase (CK) and lactate dehydrogenase (LDH) were evaluated through assay kits. It was observed that simvastatin treated group showed significantly elevated CK levels (498.5±2.65 U/L) as compared to normal control group (317.1±1.7

U/L) ($p<0.0001$). The findings are similar to a study where in administration of simvastatin 80 mg/kg/day for 12 days increased plasma CK levels 315-fold above control (5, 22). Formoterol fumarate along with simvastatin administration (312.6±6.7 U/L) significantly reduced CK levels as compared to disease control group (498.5±2.65 U/L) ($p<0.0001$). Results of LDH assay showed that simvastatin treated group had significantly higher LDH levels (784.4±6.8 U/L) as compared to the saline treated group (618.1±3.3 U/L) ($p<0.001$). One study demonstrated the skeletal muscle cell injury by atorvastatin at a dose of 10 mg/kg/day as revealed by increased level of plasma LDH and CK enzyme (23). Treatment control group (588.8±15.2 U/L) showed significant decrease in the LDH level as compared to disease control group (784.4±6.8 U/L) ($p<0.0001$). Results of reduced CK and LDH levels suggest that dose or duration of formoterol fumarate if increased could give better outcomes which need to be investigated.

Following sacrifice biceps femoris muscle of the left hind limb of the animal was dissected and processed for histological study. Biceps femoris muscle is composed of approximately 70% type IIB fibers (15). Statins predominantly affect type IIB fibres (15). Histological evaluation of biceps femoris showed that statin induced degenerative changes in muscle in the form of separation of myofibrils, loss of cross striation, enlarged edematous muscle fibers and central nucleation. While formoterol fumarate treated group showed reduced degeneration of tissue and less necrosis. Parallel findings in terms of histological study were observed to the effect of simvastatin producing necrosis in biceps femoris muscle (5).

Wannenes et al., mentioned that formoterol fumarate transcriptional up regulation or down regulation atrophy-related genes are characteristic feature of muscle atrophy; the most relevant atrogenes are 2 ubiquitin ligases named atrogin-1/Mafbx and MuRF1 and cell growth in C2C12 cell line and also revealed that formoterol fumarate concentrations of at least 10^{-6} M augment rates of protein accretion and may represent for enhanced cell growth (9). Hence, in the present study formoterol fumarate at 10^{-6} M was used for in-vitro cell line MTT assay. Simvastatin inhibited Akt phosphorylation and were cytotoxic to C2C12 cell line at 10 µM for 24 hr (24); hence in the present study simvastatin at the dose of 10 µM was used for 24 hrs following incubation period. Simvastatin treated cells showed significant decrease in cell viability as compared to normal treated cells ($p<0.001$). While formoterol fumarate along with simvastatin showed significant increase in cell viability ($p<0.001$).

The above findings demonstrate beneficial effects of formoterol fumarate on simvastatin induced myopathy. The effects are probably related to anti atrophy or anti myopathic activity via inhibition of myostatin activated by the Akt/mTOR pathway responsible for protein synthesis.

Conclusion

Simvastatin-induced myalgia in rats was reverted by administration of formoterol fumarate as evidenced by improvement in motor co-ordination measured by Rota rod and open field activity; decreased levels of CK, LDH; improvement in muscle necrosis and on cell viability. Thus formeterol fumarate stands as an adjunct therapy for improving simvastatin induced myopathy. However the mechanism needs to be further investigated.

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