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Effect of Milnacipran and Atorvastatin Alone and In Combination In Rodent Model of Inflammatory Pain

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ABSTRACT

5-Hydroxytryptamine (serotonin) (5-HT) and norepinephrine (NE) are implicated in modulating descending inhibitory pain pathways in the central nervous system. Milnacipran (MLN) is a selective and potent dual 5-HT and NE reuptake inhibitor (SNRI). In the present study, the effects of HMG-CoA reductase inhibitor atorvastatin (ATR) on antidepressant- induced anti-nociception have been investigated. Anti-nociceptive effects were evaluated using formalin test in rats after administration of atorvastatin and milnacipran alone as well as in combination. Fifty microlitre of 2.5% formalin solution was injected subcutaneously into the plantar surface of the right hind paw and nociceptive behavior was observed up to 45 min in the blocks of 5 min. Milnacipran induced a dose-dependent anti-nociception in the first phase as well as in the second phase of the formalin test at the dose levels of 10, 20, 40 and 60 mg/kg, p.o.. Milnacipran significantly attenuated the duration of licking and licking frequency both in second phase. Atorvastatin (1, 5, 10 and 20 mg/kg, p.o.) did not inhibit the nociceptive behavior of formalin significantly when treated alone. A combination of sub-therapeutic doses of the milnacipran (20 and 40 mg/kg, p.o.) with atorvastatin (10 and 20 mg/kg, p.o.) potentiate anti-nociception induced by antidepressant significantly. It is concluded that the atorvastatin modulate the antidepressant-induced anti-nociception in formalin induced inflammatory pain model. However, studies in chronic models of neuropathic pain are required to evaluate the efficacy of milnacipran and atorvastatin combination in rats.

Keywords: Atorvastatin, Formalin Test, Inflammatory Pain, Milnacipran

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INTRODUCTION

Pain, a complex neurobiological state, involves various neurochemical factors affecting peripheral and central pain-signaling mechanisms. Different guidelines showed that antidepressant agents are the first line treatment for neuropathic pain through a number of mechanisms[1]. The neurochemical mechanisms of anti-nociceptive effects of antidepressants have not been well described. Most antidepressants increases the monoamine levels at neuronal terminals by inhibiting the reuptake of monoamines, including norepinephrine and serotonin [2]. It is presumed that higher levels of monoamines in synaptic clefts can induce anti-nociceptive effect through changes in pain threshold. However, there is still controversy over the identity of the monoamine receptors (or receptor subtypes) responsible for these analgesic effects, in addition to their location (central or peripheral) [3].

It is proven that statins have various pleiotropic effects like anti-inflammatory[4], improvement of endothelial dysfunction[5], immunomodulatory[6] and plaque stability[7]. Kwak et al 2000[8] showed that statins directly inhibit the induction of Major histocompatibility complex class II (MHC-II) expression by inhibiting interferon-γ (IFN- γ) and thus repress MHC-II mediated T cell activation. Various mechanisms are involved in anti-inflammatory properties of statins that may or may not involve the HMG-CoA reductase/ mevalonate pathway. Atorvastatin has pro-inflammatory as well as anti-inflammatory effects that are mainly independent of its effects on blood cholesterol[9]. Statins inhibit inflammatory responses in different models of autoimmune disease such as collagen- and complete Freund's adjuvant (CFA)-induced arthritis and experimental encephalomyelitis [10,11]. Atorvastatin has shown the anti-nociceptive activity in rat model of CFA-induced arthritis [11].

Taking into account the anti-nociceptive properties of antidepressants and statins in animal model of pain, the aim of this study was to investigate the anti-nociceptive effect of milnacipran and atorvastatin alone and in the combination of sub-therapeutic doses of both milnacipran and atorvastatin.

MATERIAL AND METHOD

Drugs and chemicals

Atorvastatin and milnacipran were kind gift from ONS Pharma, Rajasthan, India. Atorvastatin and milnacipran suspensions were prepared in 0.5% methyl cellulose in water for this study. Chemicals like formalin and methyl cellulose powder used for the study were of analytical grade.

Animals

Male Sprague-Dawley rats, 104 in number, weighing 190 to 250g were used in this study. The rats were housed in groups of four animals at 20°C to 25°C, in a humidity-controlled room under a 12:12-h light/dark cycle. The rats were supplied food and water *ad libitum* and were given at least 3 days to adapt to the animal room before being tested. The ambient temperature during testing was 20 to 25°C. The animals were brought to the test room at least 1 h before testing. Rats were used maidenly. All procedures were approved by our Institutional Animal Ethics Committee.

Experimental design

The whole study was performed in three sets of experiments. In each experimental set animals were divided in to five groups of 8 animals each. One group of animals was treated with vehicle alone i.e. 0.5% methyl cellulose in water. The treatment was given to the animals of respective group one hour prior to formalin injection. In the first experiment, animals were dosed orally with milnacipran alone (10, 20, 40 and 60 mg/kg) while in the second experiment, animals were dosed orally with atorvastatin alone (1, 5, 10 and 20 mg/kg).

From these two experiments, two doses of each milnacipran and atorvastatin were selected for third experiment in which animals were dosed orally with four possible combinations of milnacipran (20 and 40 mg/kg) and atorvastatin (10 and 20 mg/kg).

Formalin test

The method was followed as described by Yokogawa *et al*, 2002 with some modifications [12]. Fifty microliters of formalin solution (2.5% in water) was injected subcutaneously into the plantar surface of the right hind paw with a 30-gauge needle. The animal was immediately placed into an open Plexiglass box (30 X 30 X 34 cm) serving as the observation chamber. Following injection, the rat was observed for a 45-min period. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. The recording time was divided into 9 blocks of 5 min.

Rats usually lick the injected paw in two phases: immediately after injection (first phase) and approximately 15 min after injection (second phase). We measured the total time (in seconds) that the animal spent licking the injected paw (duration) during the period lasting from 0 to 10 min (first phase) and 16 to 45 min after formalin injection (second phase). The licking frequency was also recorded for the 45 min along with duration of licking after formalin injection.

Statistical analysis

Results are expressed as mean±SEM. The statistical significance of differences between experimental data was analyzed with analysis of variance, followed by the Dunnett's post hoc

test. Values of $p < 0.05$ were considered indicative of statistical significance.

RESULTS AND DISCUSSION

Formalin test is a widely accepted model of prolonged noxious stimulation, which is based on the assessment of behaviors induced by subcutaneous administration of aqueous formaldehyde solution (formalin) into the animal's paw [13]. Formalin-induced behaviors such as licking and biting of the injected paw are expressed as two clear-cut phases. The first phase is caused predominantly by C-fiber activation due to the peripheral stimulation [14,15] and is thought to reflect acute pain state. The second phase has been attributed to ongoing afferent input from peripheral site [16] that leads to the development of spinal cord hyper-excitability [17], and is commonly referred to as the "tonic" pain phase [18].

Earlier studies suggest that persistent inflammation leads to the hyper-excitability of dorsal horn neurons within the spinal cord, also known as central sensitization [19,20]. Central sensitization is characterized by altered responsiveness of dorsal horn neurons, expansion of receptive fields and plasticity of neuronal connections within the pain transmitting pathways leading to increased neuronal activity at supraspinal sites and to dysfunction of the endogenous spinal and supraspinal pain inhibitory mechanisms [20,21]. An imbalance of the excitatory and inhibitory mechanisms within both the ascending and descending pain inhibitory pathways could ultimately lead to persistent pain [21,22]. Thus, using 5-HT and NE reuptake inhibitor like milnacipran to restore this balance could be beneficial in persistent pain conditions.

In the present study, milnacipran (10–60 mg/kg) given orally 1 hr before the formalin injection produced a dose-dependent anti-nociceptive effect. Milnacipran (MLN) shifted the duration of licking/biting towards right in Phase-II showing that it is inhibiting the onset of nociceptive behavior induced by formalin (Figure 1). Milnacipran at doses of 20, 40 and 60 mg/kg showed statistical significant reduction of the duration of licking/biting on the acute pain state i.e. phase-I as well as in phase-II when compared to vehicle treated control group. The duration of time spent in licking/biting in phase-I and phase-II in vehicle treated control group was 58.88±3.07 and 145.13±14.59 seconds respectively. The duration of time spent in licking/biting at dose levels of 10, 20, 40 and 60 mg/kg in phase-I was 51.00±2.49, 40.88±2.38 ($p<0.01$), 30.63±1.78 ($p<0.01$) and 26.25±1.92 ($p<0.01$) seconds respectively (Figure 3c) and in phase-II was 123.38±5.37, 79.00±4.99 ($p<0.01$), 66.50 ±6.01 ($p<0.01$) and 53.13±5.40 ($p<0.01$) seconds respectively (Figure 3d).

Milnacipran induced percentage inhibition of duration of time spent in licking/biting as well as

the licking/biting frequency in phase-I and in phase-II is shown in Table 1. The results of the present study was comparable to the earlier studies where milnacipran showed the attenuation of paw licking behavior in formalin induced inflammatory pain model [23]. Milnacipran attenuated the nociceptive behavior of formalin significantly at the doses of 20, 40 and 60 mg/kg. It increased the duration of licking as well as licking frequency at these doses in first as well as in second phase. Milnacipran shifted the duration of licking towards right in second phase showing that it is inhibiting the onset of nociceptive behavior of formalin. These results were comparable to recent study where Bardin L et al. showed that reduction of formalin-induced paw licking in late phase might constitute a useful indicator of potential activity against inflammatory/centrally sensitized pain, as might be expressed in fibromyalgia [24].

Table 1: Inhibitory effect of atorvastatin, milnacipran and combination of atorvastatin and milnacipran on duration of time spent in licking/biting and on licking/biting frequency in formalin-induced inflammatory pain in rats

Treatment Groups	Dose (mg/kg)	% Inhibition			
		Duration of time spent in Licking/ Biting		Licking Frequency	
		Phase-I	Phase-II	Phase-I	Phase-II
Milnacipran	10	13.38	14.99	5.26	17.39
	20	30.57	45.56	31.58	43.48
	40	47.98	54.18	47.37	58.70
	60	55.41	63.39	57.89	67.39
Atorvastatin	10	17.83	25.32	21.05	23.91
	20	23.78	28.42	21.05	23.91
Milnacipran + Atorvastatin	20 + 10	25.90	49.35	26.32	50.00
	20 + 20	50.96	57.28	52.63	58.70
	40 + 10	50.11	60.38	47.37	63.04
	40 + 20	61.15	65.46	63.16	69.57

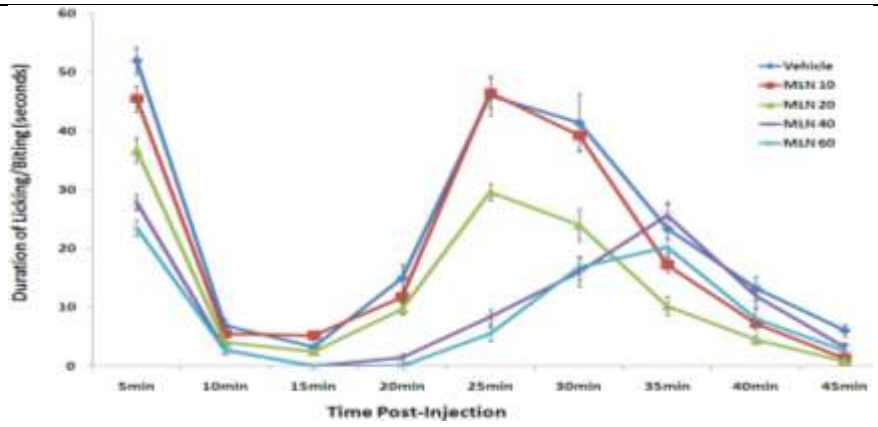

Figure 1: Dose response of milnacipran (MLN) at the dose levels of 10, 20, 40 and 60 mg/kg p.o. in formalin-induced inflammatory pain model. Data are presented as mean±SEM of duration (in seconds) of the paw licking and biting cumulatively for 5 min interval till 45 minutes after formalin injection.

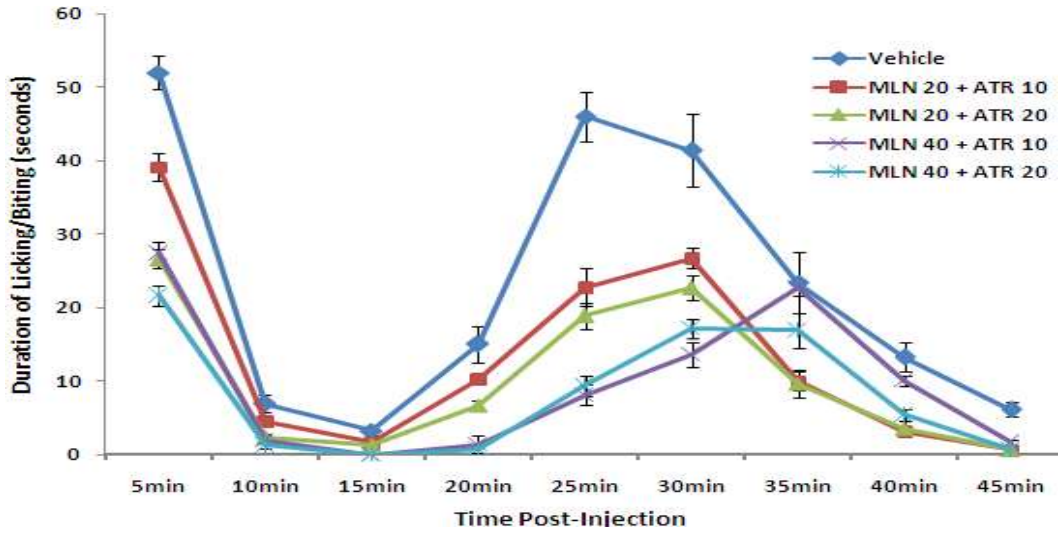

Figure 2: Anti-nociceptive effect of combination of different doses of atorvastatin (ATR) and milnacipran (MLN) in formalin-induced inflammatory pain model. Data are presented as mean±SEM of duration (in seconds) of the paw licking and biting cumulatively for 5 min interval till 45 minutes after formalin injection.

Statins have not been evaluated for their selective analgesic action in commonly used experimental models of analgesia but has been evaluated in some of the models of inflammatory pain. As reported by Barsante et al, atorvastatin attenuated the hypernociception in inflamed joints in rat model of adjuvant-induced arthritis [11]. Also in a model of mechanical hypernociception in mouse paw with an electronic pressure-meter, atorvastatin inhibited the inflammatory hypernociception after oral administration [25]. It has been reported that pretreatment with atorvastatin reduces the levels of bradykinin, tumor necrosis factor-a (TNF-a), interleukin-1b (IL-1b) the chemokines which are involved in the induction of hypernociception [25]. Some of the recent studies support the anti-nociceptive effect of atorvastatin in various animal model of pain in rat and mice [26-28]. Atorvastatin (1–20 mg/kg, p.o.) was given 1 hr before the formalin injection failed to produce dose-dependent anti-nociceptive effect. All four doses of atorvastatin (ATR) did not have any significant effect in phase-I whereas, at the doses of 10 and 20 mg/kg it decreased the duration of licking/biting significantly in phase-II when compared to vehicle treated control group. The duration of time spent in licking/biting at dose levels of 1, 5, 10 and 20 mg/kg in phase-II was 62.50, 57.03, 55.36 and 56.80% higher than phase-I (Figure 3a, 3b). Atorvastatin induced percentage inhibition of duration of time spent in licking/biting as well as the licking/biting frequency in phase-I and in phase-II is shown in Table 1. In the present study atorvastatin significantly attenuated the inflammatory pain in late phase of formalin-induced inflammatory pain model. Atorvastatin failed to produce anti-nociceptive effect induced by formalin as it did not significantly increased the duration and frequency of licking. Only higher

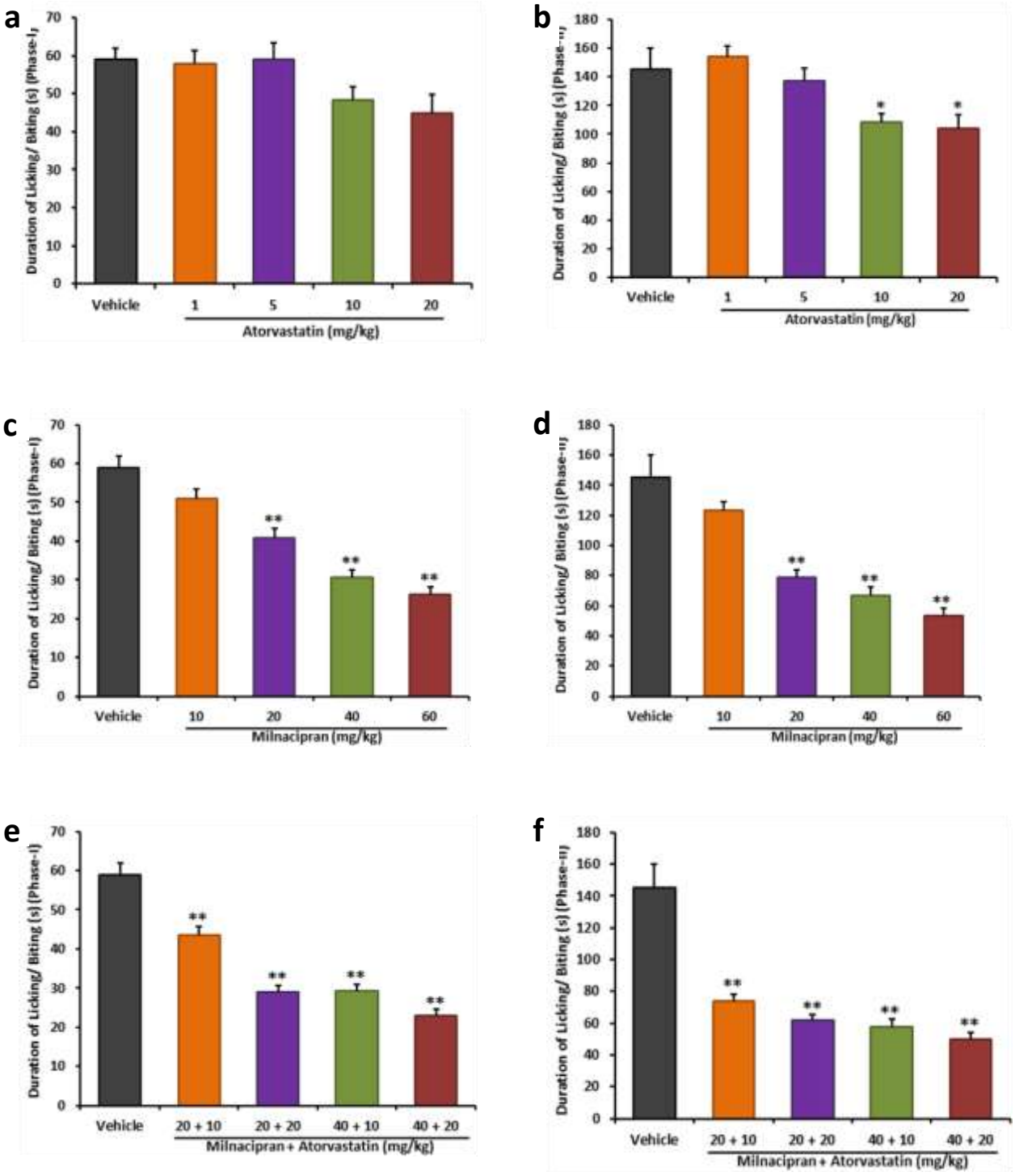

Figure 3: Anti-nociceptive effect of atorvastatin (a, b) and milnacipran (c, d) alone and in combination (e, f) in formalin-induced inflammatory pain model. Data are presented as mean±SEM of duration of licking/biting in seconds in phase-I (a, c, e) and phase-II (b, d, f) after formalin injection. *p<0.05, **p<0.01; one-way ANOVA followed by Dunnett's multiple comparison test as post hoc analysis comparing to vehicle treated control group.

dose of atorvastatin (10 and 20 mg/kg) increases the duration of licking significantly in second phase.

For the combination study two doses each of milnacipran (20 and 40 mg/kg) and atorvastatin (10 and 20 mg/kg) were selected to evaluate four possible combinations for their anti-nociceptive activity. All four combination groups of milnacipran and atorvastatin when given orally 1 hr before the formalin injection produced a significant anti-nociceptive effect. Milnacipran at 40 mg/kg in combination with atorvastatin (10 and 20 mg/kg) shifted the duration of licking/biting towards right significantly in phase-II showing that it is inhibiting the onset of nociceptive behavior induced by formalin (Figure 2). All combination groups showed statistical significant reduction of the duration of licking/biting in phase-I as well as in phase-II when compared to vehicle treated control group (Figure 3e and f). The percentage inhibition of duration of time spent in licking/biting and the licking/biting frequency in phase-I and phase-II produced by all four combination groups of milnacipran (20 and 40 mg/kg) and atorvastatin (10 and 20 mg/kg) are shown in Table 1. Atorvastatin increased the anti-nociceptive effect of milnacipran. Though it is not producing any additive effect when administered along with milnacipran but it potentiates the anti-nociceptive activity of milnacipran dose dependently. Further studies are required for in-depth knowledge of mechanism by which the atorvastatin potentiates the anti-nociceptive behavior of milnacipran.

CONCLUSION

Milnacipran attenuated the nociceptive behavior of formalin significantly at the doses of 20, 40 and 60 mg/kg. It increases the duration of licking as well as licking frequency at these doses in both phases. In phase-II, milnacipran shifts the duration of licking towards right showing that it is inhibiting the onset of nociceptive behavior of formalin. Atorvastatin failed to produce anti-nociceptive effect induced by formalin as it did not increase the duration of licking as well as licking frequency significantly. Only higher dose of atorvastatin (20 mg/kg) increased the duration of licking significantly in phase-II. Atorvastatin increased the anti-nociceptive effect of milnacipran in a dose dependent manner.

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