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Amitriptyline and Acute Inflammation: A Study Using Intravital Microscopy and the Carrageenan-Induced Paw Edema Model

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Key Words
Amitriptyline • Inflammation, acute

Abstract

Background/Aims: Antidepressants are reported to exhibit antiinflammatory effects. However, mechanisms involved in this action have not been elucidated. Thus, the objectives of the present study were (a) to evaluate the effects of amitriptyline on the acute inflammatory process, and (b) to investigate the participation of $\alpha_1$-adrenergic receptors and glucocorticoids as possible mechanisms implicated in the amitriptyline action on inflammation. Methods and Results: Single and multiple doses of amitriptyline were administered to rats submitted to the carrageenan-induced paw edema model. The results showed a significant antiedematous reaction to amitriptyline, mainly when administered at each elimination half-life. The next step was to evaluate its effects on leukocyte behavior, using intravital microscopy. Amitriptyline produced a significant effect on leukocyte behavior. To investigate possible mechanisms involved, a glucocorticoid receptor antagonist (RU-486) and an $\alpha_1$-adrenergic receptor antagonist (prazosin) were used. RU-486 administration lacked the ability to decrease the amitriptyline antiinflammatory effects in the carrageenan-induced paw edema model. Prazosin pretreatment potentiated the amitriptyline antiinflammatory effect without presenting an effect per se. Conclusion: The present study shows the ability of amitriptyline to decrease edema and affect leukocyte behavior in an acute inflammatory process; and, for the first time to our knowledge, we suggest the involvement of $\alpha_1$-adrenoceptors in the antiinflammatory effects of amitriptyline.

Introduction

Over the past decades, several studies on monoaminergic theories of depression were unable to provide a satisfactory answer to many questions about this disorder. This reality led to the development of new theories, which analyze depression in a neuroimmune perspective, i.e. as a proinflammatory process [1, 2]. Supporting this hypothesis, several studies have demonstrated antiinflammatory effects for various antidepressants, among them tricyclic amitriptyline [3–5].

Inflammation is a well-known phenomenon characterized by 5 cardinal signs, namely redness (rubor), swelling (tumor or edema), heat (calor), pain (dolor) and loss of function (functio laesa) [6]. Vascular events, i.e. vasodilation and increase in vascular permeability, are key points necessary for leukocyte arrival at the lesion site, and they are responsible for the signs of redness, heat and edema which characterize inflammation. In the edema, the cells and plasma proteins, normally restricted to the blood vessels, gain access through the postcapillary ve-
nules to the extravascular tissues at the inflammatory site. The activated endothelium of the blood vessels allows selective rolling, adhesion and extravasation of neutrophils by the expression of specific adhesion molecules, finalizing exudate formation [7].

The observation of the cardinal signal signs of inflammation is one important strategy for the assays of antiinflammatory activity. It can be observed in the classic method by Meier et al. [8], which tested for the inhibition of granuloma formed around a cotton pellet inserted subcutaneously in the rat. The biological activity of indo-methacin was discovered in this way. Winter et al. [9, 10] empirically established carrageenan as an important phlogistic agent since it induced reproducible edema which responded in a fairly specific way to nontoxic doses of antiinflammatory drugs, yielding parallel linear-log dose-response data. This discovery entails the possibility of testing small samples of new compounds and reporting results within hours [9]. Since then, Winter et al. [9, 10] proposed the inflammatory response to an injection of carrageenan into the rat paw as an animal model to evaluate acute inflammation, and several authors have considered a relationship between edema volume and inflammation severity [11].

Moorhead et al. [12] evaluated transduced mouse ears injected with first- and second-generation viruses in relation to edema, inflammatory infiltrate and transduced gene expression as a function of virus vector dose. The preterminal protein virus Ad5dl308gptpβ-gal induced edema approximately 10-fold less efficiently in the first 4 days and an inflammatory cell infiltrate more than 10-fold less efficiently at day 4 than Ad5dl308-gal. This evidence suggests a fairly good, approximately linear relationship between edema and inflammation. Abdel-Salam et al. [4], based on antiedematous results obtained by the carrageenan-induced paw edema model, confirmed the antiinflammatory effect of several antidepressants.

The production of a variety of inflammatory mediators including cytokines, histamine, serotonin, chemokines, prostaglandin and products of proteolytic cascades is responsible for cellular and vascular events of an inflammatory response. Several hormones also influence these events, such as adrenaline and glucocorticoids. Garcia-Leme and Wilhelm [13] verified that adrenalectomized animals presented an increase in histamine-induced vascular permeability, a fact reverted by corticosterone administration. Farsky et al. [14] showed that adrenalectomy or the use of a glucocorticoid synthesis inhibitor increased leukocyte rolling behavior, reinforcing the concept of an antiinflammatory action of this hormone.

Moreover, considering the influence of vascular aspects on the inflammatory phenomenon, drugs that influence the vascular diameter could interfere in edema formation. Within this context, amitriptyline, the object of the present study, in addition to inhibiting monoamine reuptake, is well known to present antimuscarinic, anti-histaminergic (H1) and α1-adrenergic antagonist effects [4, 5, 15]. The last action was related to vasodilation and consequent hypotension observed at the beginning of the treatment. On the other hand, Sugino et al. [16] verified that prazosin (α1-adrenergic antagonist; 1–3 mg/kg) produced a reduction in tumor necrosis factor (TNF)-α and interleukin (IL)-6 in lipopolysaccharide-treated mice, with a slight increase in antiinflammatory cytokine IL-10. The authors suggested a participation of α1-adreceptors in the inflammatory process. Thus, considering that α1-adrenergic antagonism induces vasodilation (a proinflammatory action) but decreases proinflammatory cytokines [16], the possible participation of α1-adrenergic receptor in the amitriptyline effect on inflammation is not clear.

Therefore, the objectives of the present study were (a) to evaluate the effects of amitriptyline on the acute inflammatory process, i.e. on carrageenan-induced paw edema and on leukocyte behavior, by intravital microscopy to confirm the direct relation between edema volume reduction and decrease in leukocyte action, and (b) to investigate the participation of α1-adrenergic receptors and glucocorticoids as possible mechanisms implicated in amitriptyline action on inflammation.

**Materials and Methods**

**Animals**

Male Wistar rats weighing 250–350 g were used. The animals were housed in plastic cages (41 × 36 × 16 cm; 5 per group) in temperature-controlled (21–23°C) and artificially lighted rooms in a 12 h/12 h light/dark cycle (lights on at 7.00 a.m.) with free access to rodent chow and water. The experiments were performed in a different room with the same temperature as the animal colony, to which the animals were transferred and maintained in their home cages 1 day before the experiments. The rats were housed and used in accordance with the guidelines of the Committee on Care and Use of Animal Resources of the School of Veterinary Medicine, University of São Paulo; these guidelines are similar to those of the United States National Institute of Health. Each animal was used once.

**Reagents**

Amitriptyline, kindly donated by Cristália Produtos Químicos e Farmacêuticos Ltda. (São Paulo, Brazil), was used at a dose of 10 mg/kg, 1 h before the injection of the phlogistic agent (λ-
carrageenan 1%; Sigma-Aldrich, St. Louis, Mo., USA), ketamine hydrochloride 10% (Dopalen®, SESPO Indústria e Comércio Ltda., São Paulo, Brazil) and xylazine hydrochloride 2% (Calmipun®; União Química Farmacêutica Nacional S.A., São Paulo, Brazil) were used for anesthesia. Saline (NaCl 0.9%; Labsynth Produtos para Laboratório Ltda., São Paulo, Brazil) was used as an amitriptyline and prazosin vehicle, and Ringer solution (Aster Produtos Médicos Ltda., São Paulo, Brazil) as the carrageenan diluent. The glucocorticoid receptor (GR) antagonist RU-486 (10.0 mg/kg; Sigma-Aldrich) dissolved in propylene glycol 5% in alcohol (70°) was used twice: 13 h and 1 h before the amitriptyline treatments. The α₁-adrenergic receptor antagonist prazosin (Minipress®; 2.0 mg/kg; Pfizer, São Paulo, Brazil) was also employed 30 min before amitriptyline injection. The time frames were chosen considering (a) the elimination half-life of amitriptyline in rats (3.25 h) [17, 18]; (b) the literature on RU-486 [19], amitriptyline [4], prazosin [20] and carrageenan [20–22], and (c) previous data from our laboratory (data not shown).

Carrageenan-Induced Paw Edema

The acute inflammation was induced by a subcutaneous injection of 100 μL of carrageenan 1% into the left hind paw of the rats, according to previous experience of the laboratory [21, 22]. The volume (in milliliters) of the induced edema was measured with the aid of a plethysmometer (Plethysmometer 7150; Ugo Basile, Varese, Italy). Measurements were made immediately before as well as 1, 2, 3, 4, 6 and 8 h after carrageenan injection to determine the differences in paw volume up to the tibiotarsal joint. The rats received the drugs or their vehicles intraperitoneally at a volume of 1.0 ml/kg body weight, 1 h before carrageenan administration.

Study of the Microcirculation ‘in vivo’ and ‘in situ’: Direct Intravital Microscopy

Leukocyte parameters were examined as previously described [23–26]. The rats were anesthetized (i.p. injection of a mixture, 1:1, of xylazine hydrochloride 2% and ketamine hydrochloride 10%), and the mesenteric tissue was exteriorized for microscopic examination in situ. The preparation was not affected by respiratory movements of the animals, and its microcirculatory characteristics remained basically invariant throughout the course of the experiment. The animals were maintained on a special board thermostatically controlled at 37°C, with a transparent stage on the face of the venular endothelium was studied in a segment of the vessel. Rolling leukocytes (rollers) were defined as leukocytes moving at a lower speed than red blood cells in the same stream. The interaction of leukocytes with the luminal surface of the venular endothelium was studied in a segment of the vessel. Rolling leukocytes (rollers) were defined as leukocytes moving at a lower speed than red blood cells in the same stream. The number of rolling leukocytes was counted at 1-min intervals during the times of 15, 30, 45 and 60 min. These leukocytes moved sufficiently slowly to allow individual visualization and counting as they rolled over a 100-μm length of a venule [27]. Adherent leukocytes were considered as such if they remained stationary for >30 s [15]. The number of adherent cells (stickers) was expressed as the number of stationary cells per 100 μm length of each venule. To assess leukocyte transmigration, the number of cells that accumulated in a 2,000 μm² standard area of connective tissue adjacent to a postcapillary venule was determined. Velocity was estimated based on the time spent to cross 100 μm of venule [24, 25, 28].

Experimental Design

Five independent experiments were performed in accordance with Good Laboratory Practice protocols and quality assurance methods. The first experiment was designed to investigate amitriptyline effects on carrageenan-induced paw edema, administered once a day during 7 or 14 days. Briefly, 40 rats were randomly distributed into 4 groups: 20 rats were treated with saline (control groups C7 and C14, treated during 7 and 14 days, respectively) and the other 20 received amitriptyline 10 mg/kg intraperitoneally for the same periods (experimental groups E7 and E14). On the last day, 1 h after the last dose, they were injected with carrageenan 1% into the left paw, and the paw volume was measured at baseline as well as 1, 2, 3, 4, 6 and 8 h later, using a plethysmometer. The same procedure was carried out for a 28-day treatment.

In a second experiment, the objective was to evaluate the effect of different protocols of treatment on the carrageenan-induced paw edema. Forty rats were randomly divided into 5 groups: a control group treated intraperitoneally with NaCl 0.9% (control i.p.; n = 5); a control group treated subcutaneously with NaCl 0.9% (control s.c.; n = 5); an experimental group treated intraperitoneally with a single dose of amitriptyline 10 mg/kg (AMI i.p.; n = 10); an experimental group treated subcutaneously with a single dose of amitriptyline 10 mg/kg (AMI s.c.; n = 10), and an experimental group that received amitriptyline 10 mg/kg intraperitoneally, administered at each elimination half-life (2.5–3 h; AMI i.p. half-life; n = 10). One hour after the treatments, they were injected with the phlogistic agent carrageenan into the left paw, following all the procedures described previously regarding the plethysmometer.

In a third experiment, the influence of amitriptyline on leukocyte behavior was investigated. Briefly, rats were randomly distributed into 2 groups: control (NaCl 0.9%; 1 ml/kg) and amitriptyline (10 mg/kg i.p.). One hour after the respective treatments, they received carrageenan (500 μg/2 ml Ringer solution, i.p., single dose.). Two hours later they were evaluated in terms of leukocyte rolling, adhesion and transmigration parameters, and the time to cross 100 μm of venule, as described above.

In order to investigate possible mechanisms involved in the amitriptyline effect, a fourth experiment was conducted, in which rats were pretreated with glucocorticoid antagonist RU-486 (10 mg/kg) or its vehicle 13 h and 1 h before the respective treatments (NaCl 0.9% or amitriptyline 10 mg/kg, administered at each elimination half-life). One hour later, they were injected with carrageenan into the left paw, and the paw volume was measured as previously described.

In a last experiment, 10 control animals were pretreated with prazosin vehicle (saline) and, after 30 min, received saline (amitriptyline vehicle; 1 ml/kg i.p.). Experimental groups were (1) pretreated with saline and, after 30 min, with amitriptyline (10 mg/kg i.p.; amitriptyline group), (2) pretreated with prazosin (2 mg/kg i.p.) and, after 30 min, with saline (prazosin group), and (3) pretreated with prazosin (2 mg/kg i.p.) and, after 30 min, with amitriptyline (10 mg/kg i.p.; prazosin-amitriptyline group). One
hour later, animals of all the groups received carrageenan in the left paw for the plethysmometry procedures. In this fifth experiment, all groups received amitriptyline or its vehicle at each elimination half-life (2.5–3 h). The schematic protocol is presented in figure 1.

**Statistical Analysis**

Results were first tested for homoscedasticity by the Bartlet test. Parametric data were then analyzed by a t test or one-way or two-way analyses of variance (ANOVA), followed, when necessary, by the Tukey-Kramer test for multiple comparisons. Kruskal-Wallis or Newman-Keuls nonparametric tests were performed when necessary. The level of significance was set at p < 0.05 for all comparisons made. The Sigma Stat 3.0® software was used throughout to analyze the data, which were expressed as means ± SD.

**Results**

**Effects of Long-Term Amitriptyline Administrations on Carrageenan-Induced Paw Edema**

Data obtained from amitriptyline administration during 7 and 14 days were analyzed, and a two-way ANOVA indicated statistically significant differences between treatments (F3, 192 = 30.104; p < 0.001) and times (F5, 192 = 75.569; p < 0.001) and an interaction between these factors (F15, 192 = 2.12; p < 0.05). A Tukey post hoc test showed that amitriptyline administered for 7 (E7) or 14 (E14) days produced a significant reduction in the paw edema in the times of 1 h (p < 0.01 and p < 0.05, respectively), 2 h (p < 0.001 and p < 0.01) and 3 h (p < 0.001 and p < 0.05) when compared to the respective controls (C7 and
In the fourth hour, only amitriptyline administered during 7 days was able to decrease the paw edema volume in a significant way (p < 0.01). There were no significant differences between E7 and E14 data (p > 0.05). When the data obtained from amitriptyline administration during 28 days were analyzed, a two-way ANOVA showed significant differences between the treatments (F1, 90 = 52.798; p < 0.001), times (F5, 90 = 49.962; p < 0.001) and an interaction between these factors (F5, 90 = 9.805; p < 0.001). A Tukey test indicated that amitriptyline administered during 28 days (E28) significantly reduced the paw edema in the times of 1, 2 and 3 h (p < 0.001) when compared to the control (C28) group. These results are depicted in figure 2.

**Influence of Different Administration Protocols on Antiinflammatory Amitriptyline Effects**

Data obtained from a control group treated with a single intraperitoneal saline dose (control i.p.; n = 5) and from a control group treated with a single subcutaneous saline dose (control s.c.; n = 5) were compared and differences between them were not found for any of the times analyzed (p > 0.05). Thus, these data were pooled for statistical analysis and interpretation. The experimental groups were treated with a single intraperitoneal amitriptyline dose (10 mg/kg; AMI i.p.; n = 10) or a single subcutaneous amitriptyline dose (10 mg/kg; AMI s.c.; n = 10), and another experimental group received amitriptyline 10 mg/kg intraperitoneally, given at each elimination half-life (2.5–3 h; AMI i.p. half-life; n = 10). A two-way ANOVA showed a significant difference among treatments (F3, 216 = 42.337; p < 0.001) and times (F5, 216 = 35.307; p < 0.001), without interaction between the factors (F5, 216 = 0.667). The Tukey post hoc test application showed a decrease in paw edema volume in the first hour after carrageenan injection, in relation to the control.
group, in animals of the AMI i.p. half-life (p < 0.001) and AMI s.c. groups (p < 0.005). A significant decrease in the paw edema, in relation to the control group (p < 0.05), was observed 2 and 3 h after carrageenan injection in the groups that received amitriptyline treatments. However, only the AMI i.p. half-life group presented a decrease in the edema for every time evaluated, i.e. 1, 2, 3, 4, 6 (p < 0.01) and 8 h (p < 0.01), when compared to the control group, as depicted in figure 3.

**Amitriptyline Effects on Leukocyte Behavior Evaluated by Intravital Microscopy**

Concerning amitriptyline effects on leukocyte behavior, a t test showed differences between the groups in relation to rolling at 0 min (t = 2.406; p < 0.05), 15 min (t = 2.442; p < 0.05), 30 min (t = 2.751; p < 0.02), 45 min (t = 3.182; p < 0.01) and 60 min (t = 2.567; p < 0.05; control: n = 9; amitriptyline: n = 8). Amitriptyline also reduced leukocyte adhesion at 45 min (t = 2.483; p < 0.05) and 60 min (t = 2.616; p < 0.05; control: n = 9; amitriptyline: n = 7) and transmigration after 1 h of evaluation (t = 2.864; p < 0.02; n = 9 per group). The data obtained are depicted in figure 4. There was no significant difference in the time spent to cross 100 μm of venule (p > 0.05; control: n = 11; amitriptyline: n = 9).

**Influence of RU-486 on Antiinflammatory Amitriptyline Effects**

A two-way ANOVA showed a significant difference among treatments (F = 50.64; p < 0.001) and times (F = 28.44; p < 0.001), without interaction between the factors (F15, 180 = 0.9; p > 0.05). Amitriptyline treatment given at each elimination half-life produced a significant reduction in paw edema volume at 2, 3, 4 (p < 0.001), 6 (p < 0.005) and 8 h (p < 0.005) after carrageenan injection when compared to the control data. RU-486 produced no effects in the inflammatory process and did not revert the antiinflammatory effect of amitriptyline (p > 0.05), as can be seen in figure 5a.

**Influence of Prazosin on Antiinflammatory Amitriptyline Effects**

The data obtained from the experiment with prazosin were evaluated by two-way ANOVA, which showed a significant difference among treatments (F3, 198 = 27.426; p < 0.001) and times (F5, 198 = 29.778; p < 0.001), without interaction between the factors (F15, 198 = 1.558; p > 0.05). The Tukey test showed that amitriptyline produced a significant reduction in paw edema volume at 2 and 3 h (p < 0.005) when compared to data of the control ani-
mals. Prazosin per se did not produce an effect on the paw edema ($p > 0.05$). Animals that received prazosin and amitriptyline presented a significant decrease in the inflammatory edema at $1 (p < 0.05), 2, 3 (p < 0.001), 4 (p < 0.05)$ and $8$ h ($p < 0.05$) when compared to the control group, as represented in figure 5b.

**Discussion**

The first objective of the present study was to evaluate the effect of long-term amitriptyline administration on the acute inflammatory process. The results obtained corroborate those in the literature, showing an antiinflammatory effect for this drug [4, 29–31]. However, independently of the treatment period (7, 14 or 28 days), this effect was not observed after the fourth hour of evaluation. According to other studies [17, 18], the elimination half-life of amitriptyline in rats is around 3.25 h, while in humans, it is said to be around 24 h. In fact, the administration of amitriptyline each 2.5–3 h produced, in most of the experiments, a significant reduction in edema volume until 6 or 8 h of evaluation. These data suggest the importance of the plasmatic levels of amitriptyline or of its metabolites for the antiinflammatory effect now being reported.

The next step was to evaluate the amitriptyline effects on leukocyte behavior, using intravital microscopy. Our results showed that single-dose amitriptyline administration was not able to modify leukocyte behavior in a noninflammatory condition (data not shown). However, in a carrageenan-induced inflammatory process, amitriptyline produced a significant decrease in both leukocyte rolling, adhesion and transmigration, without modifying the time spent by these cells in rolling a venule segment. This result confirmed the direct relation between decrease in edema volume and reduction in leukocyte action. These measured parameters are known to be dependent on the expression of adhesion molecules by the endothelium and leukocytes, which is influenced by several mediators such as lipopolysaccharides, cytokines like IL-1 and TNF-$\alpha$, and mast cell products, mainly histamine [32]. Many studies have shown antidepressant effects in reducing proinflammatory cytokines [33–35]. Obuchowicz et al. [36] showed the ability of amitriptyline to decrease the production of IL-1$\beta$ and TNF-$\alpha$ in glial cell cultures of rats. Thus, taken together, the literature and the present data suggest that amitriptyline effects on leukocyte behavior might be attributable to the ability of antidepressants to decrease proinflammatory cytokines and, consequently, adhesion molecule expression and/or affinity by their ligands [37]. In fact, data from our laboratory (data not shown) confirm the ability of amitriptyline to reduce TNF-$\alpha$ and IL-1$\beta$ levels in the serum of rats.

The next question to be analyzed was related to the possible involvement of glucocorticoid hormones in the antiinflammatory effects observed. In the present study, the GR antagonist RU-486 lacked the ability to decrease the antiinflammatory amitriptyline effects on carrageenan-induced paw edema. Glucocorticoids are important hormones in the control of an inflammatory process,
hence their clinical use as steroidal antiinflammatories. Cavalcanti et al. [38] demonstrated that endogenous glucocorticoids, via activation of GR on neutrophils, physiologically control the rolling behavior of these cells and, by modulating endothelial functions, affect their adhesiveness. Weber et al. [39] evaluated the effects of some antidepressants on plasma/brain corticosterone distribution. Their results showed that amitriptyline was not able to affect brain or plasmatic levels of corticosterone in mice, whether administered in a single dose or for 2 weeks. This lack of interference may be attributed to the amitriptyline ability to antagonize 5-HT<sub>2</sub> receptors, which are responsible for the serotonergic modulation of the hypothalamic-pituitary-adrenal axis. However, the effects of a 24-hour coincubation of LMCAT cells with dexamethasone and amitriptyline, clomipramine, paroxetine, citalopram or fluoxetine were studied. All antidepressants, except fluoxetine, enhanced GR-mediated gene transcription [40]. Thus, a possible influence of glucocorticoid hormones on antiinflammatory amitriptyline effects cannot be discarded.

The role of α<sub>1</sub>-adrenergic receptors in the inflammatory response was also investigated, considering the amitriptyline actions on α<sub>1</sub>-adrenoceptors. Prazosin pretreatment potentiated the antiinflammatory effect of amitriptyline, without having an effect per se. This finding was unexpected, considering that α<sub>1</sub> antagonism induces vasodilation, an important event in the inflammatory process, which is the basis for the clinical use of prazosin as an antihypertensive drug. However, considering the findings of Sugino et al. [16], where prazosin administration produced a significant decrease in the serum levels of proinflammatory cytokines TNF-α and IL-6, and the data from the literature showing the ability of many antidepressants to reduce proinflammatory cytokines [33–35] – confirmed by previous experiments in our laboratory (data not shown) –, the present results suggest a participation of α<sub>1</sub>-adrenergic receptors in the antiinflammatory effects of amitriptyline.

Another study evaluated amitriptyline-prazosin interaction in mice submitted to the hot-plate model. As expected, amitriptyline 10 mg/kg produced an analgesic effect. Prazosin (0.1, 0.2, 0.5 and 1.0 mg/kg) was not able to promote analgesia per se, but when associated with amitriptyline, the lower doses potentiated its analgesic effect. These results suggest that the potentiation of effects between these two drugs is not restricted to the inflammatory process; it also implicates that there may be a common pathway between the antiinflammatory and analgesic mechanisms of amitriptyline [41].

In summary, the present findings show antiinflammatory amitriptyline effects both in the carrageenan-induced paw edema model and on leukocyte behavior evaluated by intravital microscopy. And, for the first time to our knowledge, we demonstrated the involvement of α<sub>1</sub>-adrenoceptors in the antiinflammatory amitriptyline effects, probably influencing proinflammatory cytokines. We believe that a better comprehension of the antiinflammatory effects of amitriptyline might shed some light on the inflammation hypothesis on depression.

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Amitriptyline and Inflammation


