Alginate and Its Comonomer Mannuronic Acid: Medical Relevance as Drugs

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Contents

Abstract The therapeutic and anti-inflammatory properties of sodium alginate and one of its components, β-D-mannuronic acid, were tested in various experimental models. On the basis of its chemical structure, β-D-mannuronic acid had been conceived as an anti-inflammatory drug. β-D-Mannuronic acid is a constituent of alginate but is not available commercially; hence, polymannuronate was obtained from an epimerase (AlgG) negative mutant of *Pseudomonas* sp. and the monomer was isolated by acid hydrolysis. The oral and intraperitoneal administration of alginate gels in experimental models of ulcerative colitis and glomerulonephritis as well as the oral administration and intraperitoneal injections of β-D-mannuronic acid in different models of rheumatoid arthritis, multiple sclerosis, glomerulonephritis, and nephrotic syndrome showed that alginate as well as β-D-mannuronic acid are able to exhibit

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their therapeutic efficacy in inflammatory diseases. Recent experimental evidence has revealed that β-D-mannuronic acid represents a novel nonsteroidal anti-inflammatory drug with a low molecular weight and which provides a new therapeutic option in attenuation of inflammatory reactions and autoimmune diseases.

1 Introduction

Alginates are natural macromolecules composed of β-D-mannuronate and α-L-guluronate linked by 1→4 glycosidic bonds. They are synthesized by bacteria and/or brown seaweeds, and the mannuronate residues of the bacteria, but not seaweed polymers, are acetylated at positions 0–2 and/or 0–3 to a variable extent (Skjak-Braek et al. 1986; Bucke 1987; Rehm 1998).

The variability in monomer block structures and acetylation which are associated with the source of alginate strongly affect the physicochemical and rheological properties of the alginate polymer (Rehm and Valla 1997).

The alginate gels are well known as being biocompatible, degradable, and nontoxic; thus, they are widely used as carriers for drug delivery, hemostatic wound dressing, and immunoisolation systems for transplantation using uncoated alginate microspheres and devices anastomosed to the vascular system as arteriovenous shunts such as alginate-impregnated polyester vascular graft (Odell et al. 1994; Agren 1996; Murata et al. 2007; Lee et al. 1997; Efentakis and Buckton 2002; Li et al. 2006; Selmi et al. 2008; Sevgi et al. 2008). On the other hand, inhibitory effects of various types of alginic acid on hyaluronidase and mast cell degranulation were examined, and it was found that alginic acid with a mannuronate to guluronate ratio of 1.0 exhibited the strongest inhibition of both activities (Asada et al. 1997). Moreover, the protective and reparative effects of sodium alginate on radiation stomatitis and suppression of radioactive absorption by this compound in animals and human subjects were investigated (Hasegawa et al. 1989; Oshitani et al. 1990; Gong et al. 1991; McGlashan et al. 2009). The effect of alginic acid on an allergy model was analyzed by anaphylaxis, a histidine decarboxylase assay, and a histamine assay. Alginic acid dose dependently inhibited systemic anaphylaxis and passive cutaneous anaphylaxis and also decreased histamine release from serum and peritoneal mast cells. All these effects were stronger than those of disodium cromoglycate, the reference drug tested. In addition, alginic acid significantly inhibited the expression level of nuclear factor (NF)-κB and some proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor α (TNF-α) (Jeong et al. 2006). Although (Yang and Jones 2008) have reported that sodium alginate causes innate immune responses through NF-κB activation, the majority of investigations have demonstrated the biocompatibility and anti-inflammatory effects of alginate and one of its components, β-D-mannuronic acid (M2000), in various experimental models and/or patients (Siebers et al. 1997; Kammerlander and Eberlein 2003; Ohsumi et al. 2005; Hori et al. 2008). In this chapter, our aim is to show the

tolerability and therapeutic potency of alginate and M2000 in different disease models as well as various pharmaceutically relevant investigations.

2 Therapeutic Effects of Alginate on Experimental Acute Ulcerative Colitis

The potential therapeutic effect of low-viscosity sodium alginate (LVA) was studied in a rat model of acute colitis induced by intracolonic administration of acetic acid (Mirshafiey et al. 2005a). To induce the experimental model of acute ulcerative colitis, the method of (Morris et al. 1989) and (Yamada et al. 1991) was used. This experimental model produced a significant ulcerative colitis. Induction of colitis also significantly enhanced the levels of serum and colonic mucosal cytokines (interleukin-6, IL-6; and TNF-α) and eicosanoids (leukotriene B_4, LTB4; and prostaglandin E_2, PGE2), which paralleled the severity of colitis. For treatment, LVA was dissolved in water and adjusted to a concentration of 0.5% (w/v). The prophylactic and therapeutic rat groups received 0.5% LVA solution as drinking water ad libitum for 7 days. The onset of oral LVA administration for prophylactic and therapeutic groups was 24 h before and after the induction of colitis, respectively. The rats were killed at the end of the week.

The results showed that intracolonic administration of 4% acetic acid resulted in acute inflammatory bowel disease (IBD) in control nontreated rats. The control rats developed colonic macroscopic damage such as diffuse hyperemia and ulcerations. Daily oral treatment with LVA (drinking 35.5 ± 2.8 ml of 0.5% w/v LVA solution per rat, which was equivalent 0.65–0.77 mg kg^{-1} LVA per day) significantly reduced the colonic damage score in prophylactic and therapeutic rat groups (with mean scores of 5.0 and 4.6, respectively) when compared with control rats (mean score 9.4; $P < 0.05$). The microscopic examination of colonic specimens revealed a marked infiltration of inflammatory cells into both mucosa and submucosa, ulceration, and fibrosis. The most pronounced lesions were observed in a nontreated (control) group. Oral administration of LVA significantly inhibited acetic acid induced mucosal injury in both prophylactic and therapeutic groups in comparison with control rats (with mean scores of 4.2 and 4.2 vs 10.3, respectively; $P < 0.05$). Moreover, the data obtained showed that LVA therapy could significantly reduce serum and colonic mucosal IL-6, TNF-α, LTB4, and PGE2 levels in treated groups compared with nontreated controls.

Figure 1 shows that LVA therapy is able to reduce significantly the serum level of TNF-α in prophylactic and therapeutic groups compared with control rats ($P < 0.001$); however, although there was a significant difference between prophylactic and control groups in the serum IL-6 level ($P < 0.003$), the difference between therapeutic and control groups was not significant ($P < 0.058$). Moreover, as shown in Fig. 2, LVA administration could significantly diminish the serum concentration of eicosanoids (LTB4 and PGE2) in prophylactic and therapeutic groups compared

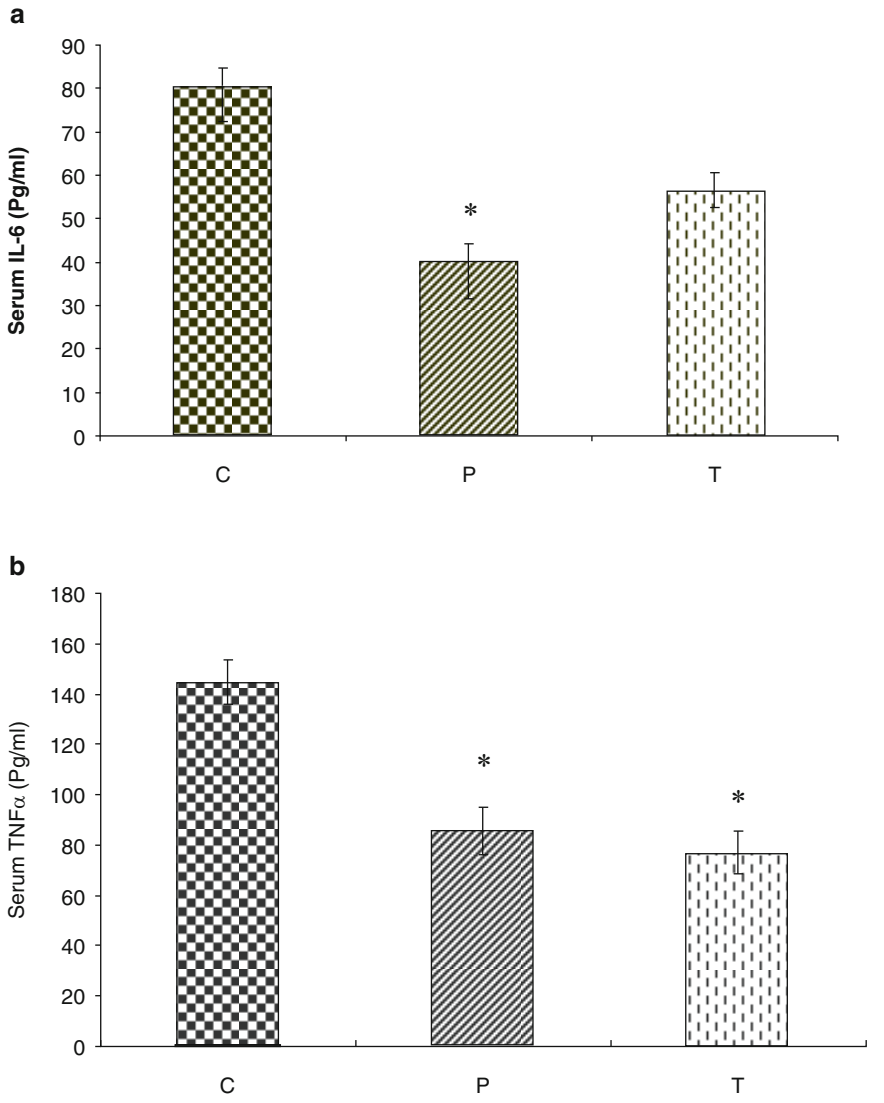

Fig. 1 Effects of low-viscosity sodium alginate (LVA) on serum levels of interleukin-6 (IL-6) (**a**) and tumor necrosis factor α (TNF-α) (**b**), 1 week after induction of colitis. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± the standard deviation (SD). *Asterisks* denote a significant difference between C and P and/or C and T groups, $*P \leq 0.05$ (Mirshafiey et al. 2005a)

with the control group ($P < 0.01$). On the other hand, with use of the supernatant of homogenized colonic tissue samples, it was found that there was a significant difference between prophylactic and therapeutic groups compared with the control group in connection with the amounts of IL-6, TNF-α, LTB4, and PGE2 ($P < 0.05$).

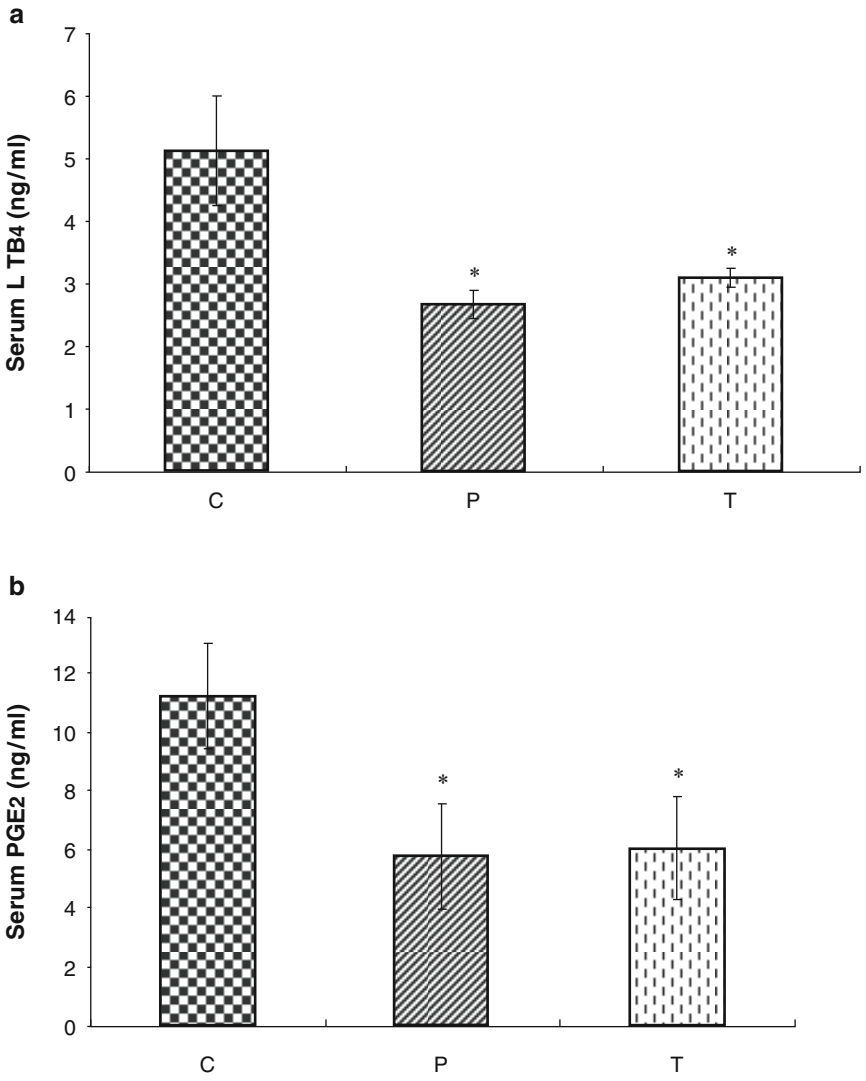

Fig. 2 Effects of LVA on serum levels of leukotriene B_4 (LTB4) (**a**) and prostaglandin E_2 (PGE2) (**b**), 1 week after induction of colitis. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $^*P \leq 0.05$ (Mirshafiey et al. 2005a)

Figures 3 and 4 show the effect of a therapeutic protocol on the reduction of the production of cytokines (IL-6 and TNF-α) and eicosanoids (LTB4 and PGE2). Taken together, the results of this study demonstrate that LVA therapy palliates the progression of colonic inflammatory lesions in an experimental model of IBD. The

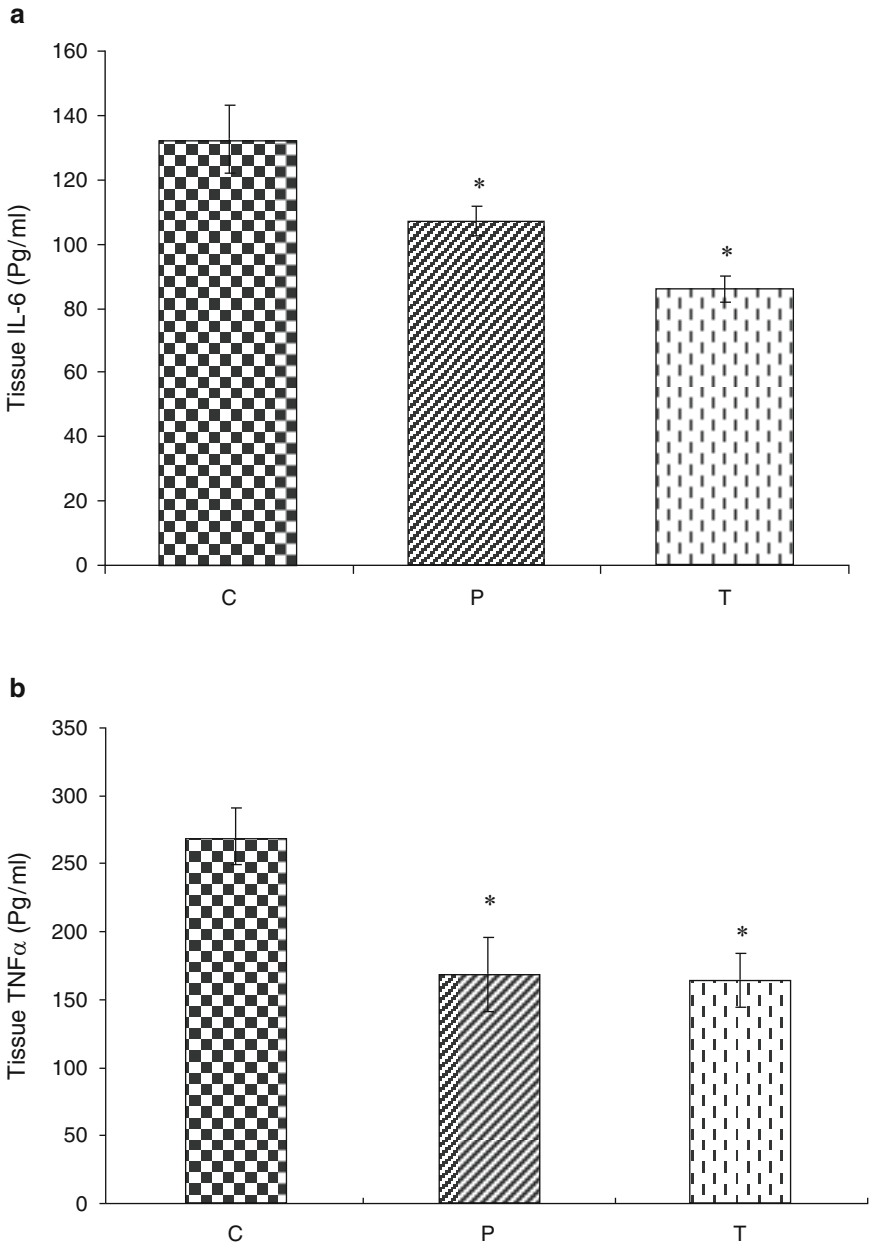

Fig. 3 Amounts of IL-6 (**a**) and TNF-α (**b**) in colonic homogenate following 1 week of oral administration of LVA. *C* control rats, *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $*P \leq 0.05$ (Mirshafiey et al. 2005a)

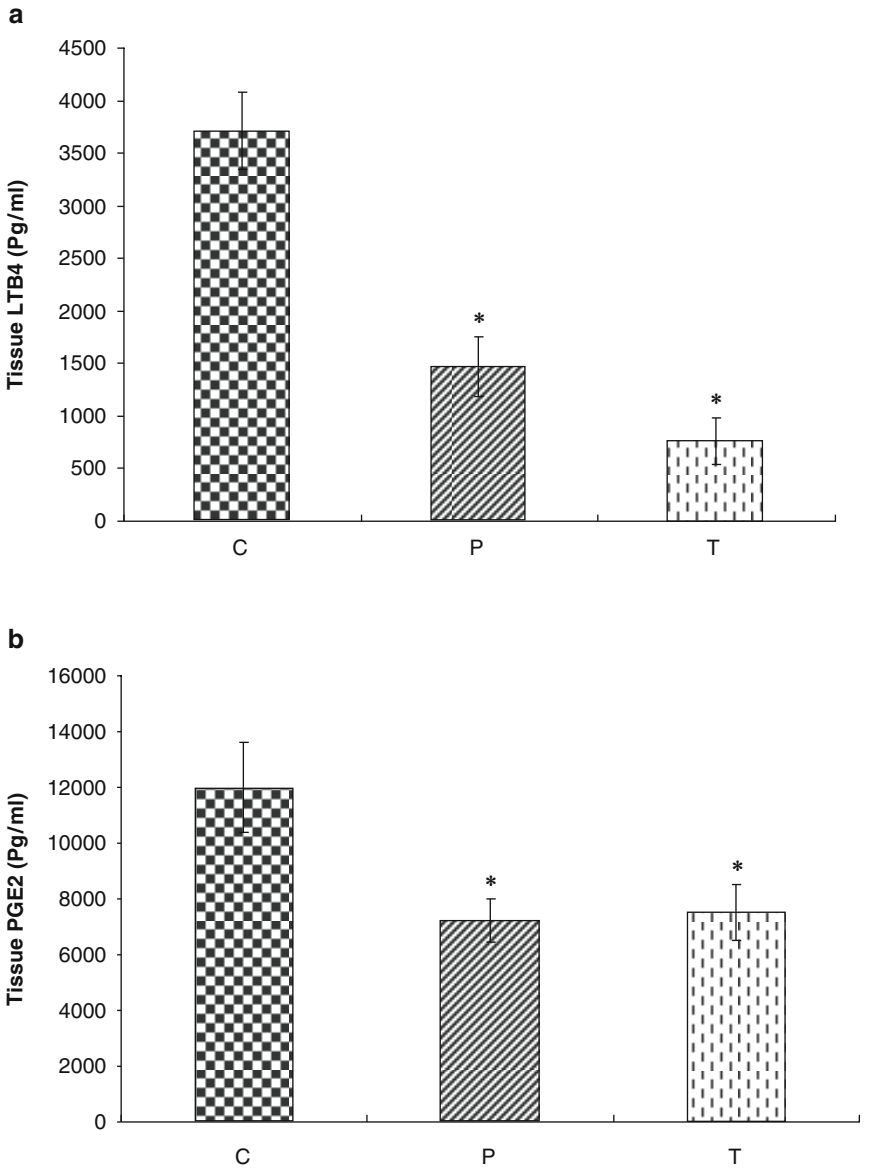

Fig. 4 Amounts of LTB4 (**a**) and PGE2 (**b**) in colonic homogenate following 1 week of oral administration of LVA. *C* Control rats *P* prophylactic group rats, *T* treated rats. Each *bar* represents the mean ± S.D. *Asterisks* denote a significant difference between C and P and/or C and T groups, $*P \leq 0.05$ (Mirshafiey et al. 2005a)

beneficial effect of LVA is associated with a reduction of cytokine (IL-6 and TNF-α) and eicosanoid (LTB4 and PGE2) synthesis. Thus, LVA as a new therapeutic option can be recommended for IBD preclinical trials.

3 Treatment of Experimental Chronic Ulcerative Colitis with Sodium Alginate

The purpose of this study was to test the therapeutic efficacy of sodium alginate in a rat model of trinitrobenzene sulfonic acid (TNBS) induced IBD (Razavi et al. 2008). This experiment was carried out using 77 Sprague-Dawley rats which were divided into five groups: normal, control, prophylactic, therapeutic, and experimental groups. The rats were killed 1, 2, 3, and 6 weeks after induction of colitis. The severity of colitis was graded macroscopically and assessed using serum and colonic mucosal cytokines and eicosanoids. Intrarectal TNBS (30 mg) produced a significant chronic ulcerative colitis, on the basis of the method of (Menozzi et al. 2006). The lesions were most severe on the seventh day after TNBS instillation, and then declined, but lesions were still observed after 6 weeks. The onset of oral LVA administration for prophylactic and therapeutic groups was 24 h before and after the induction of colitis, respectively. Macroscopic findings showed that intracolonic administration of TNBS resulted in acute (week 1) and chronic (week 6) IBD in control nontreated rats. The control rats developed colonic macroscopic damage such as diffuse hyperemia and ulcerations. Daily oral treatment with LVA (drinking 35.5 ± 2.8 ml of 0.5% W/V LVA solution per rat, which was equivalent 0.65–0.77 mg kg^{-1} LVA per day) significantly reduced the colonic damage score in prophylactic and therapeutic groups in weeks 1, 2, 3, and 6 after induction of colitis. TNBS administration also significantly enhanced the levels of serum and colonic mucosal cytokines (TNF-α and IL-6) and eicosanoids (LTB4 and PGE2), which paralleled the severity of colitis, whereas pretreatment (in the prophylactic group) and treatment with LVA were significantly able to reduce the colonic damage score, serum level, and colonic mucosal production of TNF-α, IL-6, LTB4, and PGE2 in prophylactic and therapeutic rat groups compared with nontreated controls.

Figure 5a shows that LVA therapy was significantly able to reduce the serum level of TNF-α in prophylactic and therapeutic groups (during weeks 2, 3, and 6) compared with colitis induced in control rats ($P < 0.05$). Figure 5b represents a significant difference in the serum level of IL-6 in prophylactic and therapeutic groups (during weeks 1, 2, 3, and 6) compared with control rats ($P < 0.05$). Moreover, as shown in Fig. 6a, LVA administration could significantly diminish the serum concentration of LTB4 (during weeks 1, 3, and 6) in the prophylactic group and (during weeks 1 and 3) in the therapeutic group compared with the colitis-induced group ($P < 0.05$). Figure 6b shows that LVA therapy was significantly able to reduce the serum level of PGE2 in the prophylactic group (during week 6) and in the therapeutic group (during weeks 1 and 3) compared with colitis-induced rats ($P < 0.05$). In addition, with use of the supernatant of homogenized colonic tissue samples, it was found that there is a significant difference between prophylactic and therapeutic groups compared with the colitis-induced group in connection with the amounts of IL-6, TNF-α, and LTB4 (during weeks 1 and 6) (Figs. 7a, b, 8a), whereas there was no significant difference between prophylactic and therapeutic groups compared with the control group in colonic tissue PGE2 level following LVA therapy in an experimental model of colitis (Fig. 8b). Collectively, the data showed that LVA therapy is able to suppress chronic ulcerative colitis in an experimental model.

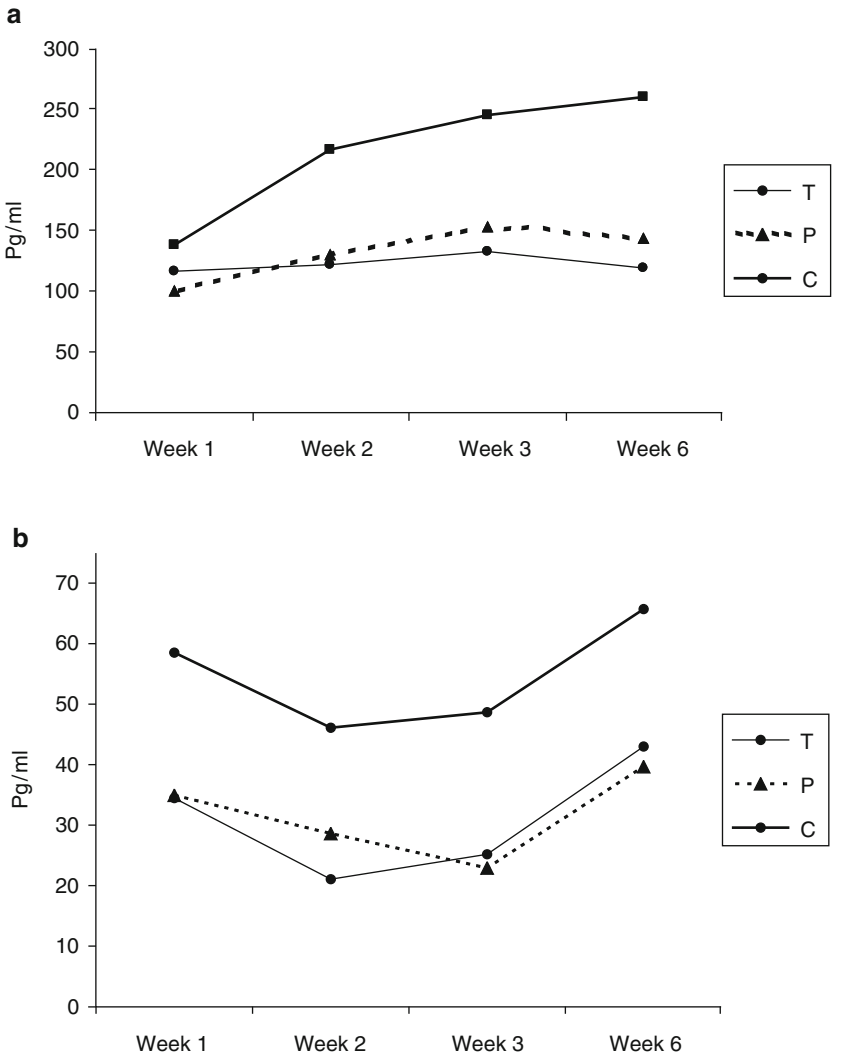

Fig. 5 Effects of LVA on serum levels of TNF-α (**a**) and IL-6 (**b**) during weeks 1, 2, 3, and 6 after induction of colitis. *C* colitis-induced rats; *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

4 Treatment of Experimental Immune Complex Glomerulonephritis by Sodium Alginate

The therapeutic efficacy of sodium alginate was studied in experimental immune complex glomerulonephritis (Mirshafiey et al. 2005). On the basis of the method of (Yamamoto et al. 1978), bovine serum albumin (BSA) nephritis as an

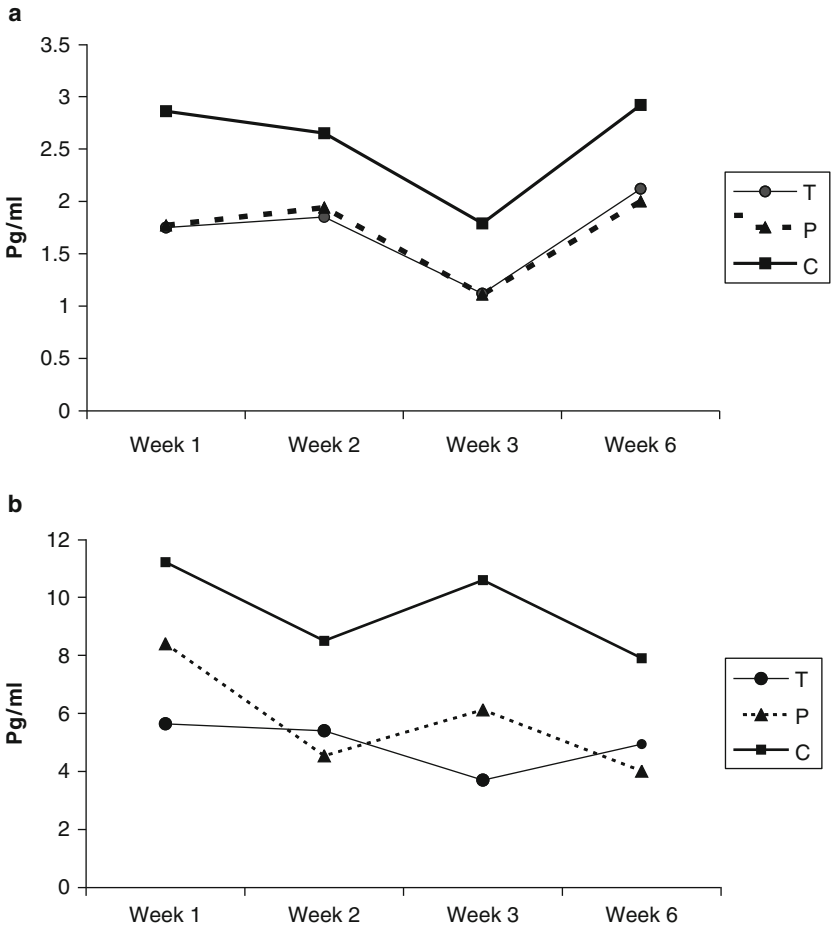

Fig. 6 Effects of LVA on serum levels of LTB4 (**a**) and PGE2 (**b**), 1 and 6 weeks after induction of colitis. *C* colitis-induced rats; *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

experimental model of glomerulonephritis was induced in rats by a subcutaneous immunization and daily intravenous administration of BSA. Sodium alginate at two different doses (25 and 50 mg kg^{-1}) was administered intraperitoneally to the treatment groups (T1 and T2) at regular 72-h intervals for 6 weeks. The onset of treatment was on day 42. Urinary protein was measured weekly and serum anti-BSA antibody was assessed by ELISA at different intervals. Rats were euthanized at the 12th experimental week and blood samples and kidney specimens were obtained. Blood urea nitrogen (BUN), serum creatinine, and serum cholesterol and triglyceride were measured at the time of death. Kidney specimens were processed for light and immunofluorescence microscopy examination.

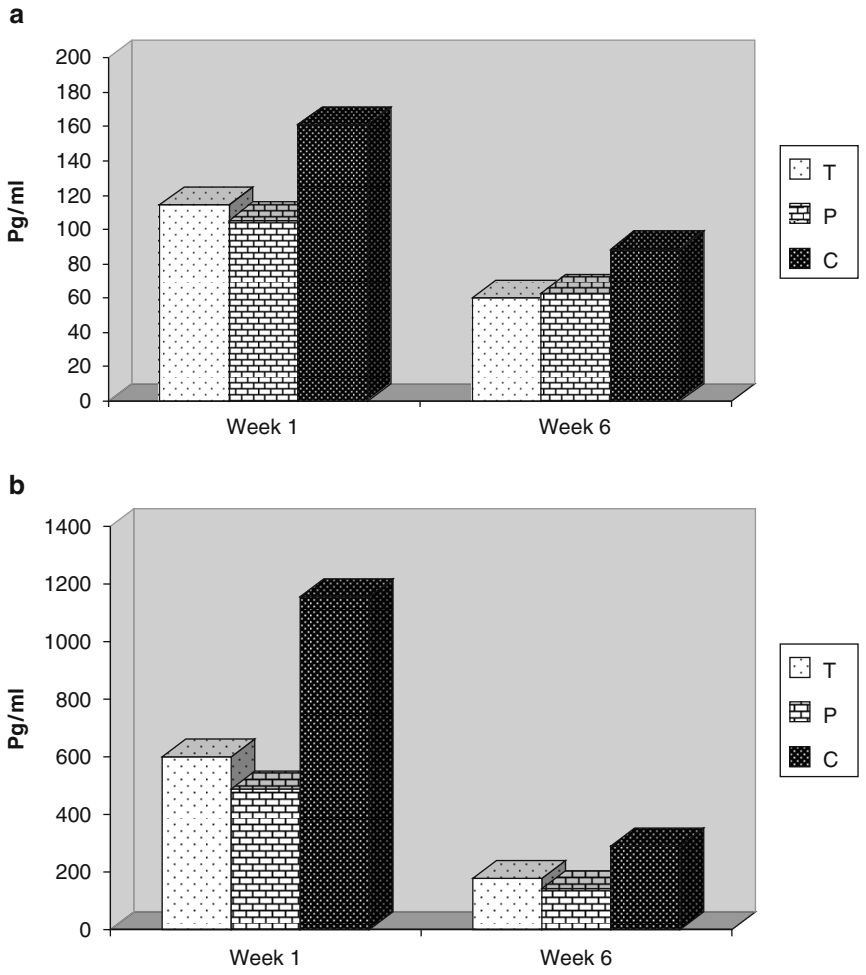

Fig. 7 Amounts of IL-6 (**a**) and TNF-α (**b**) in colonic tissue homogenate following 6 weeks of oral administration of LVA. *C* colitis-induced rats, *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

The results of this experiment showed that treatment with sodium alginate could significantly reduce the urinary protein excretion and serum creatinine in treated rats compared with nontreated controls.

Figure 9 shows the changes of the mean levels of urinary protein excretion between normal, patient, and treatment (T1 and T2) groups. The urinary protein excretion was significantly less at the end of the experiment (day 85) in LVA-treated rats (group T2, 50 mg kg^{-1}) than in the nontreated controls. Comparison of BUN and serum creatinine concentrations among various groups showed only significant reduction ($P < 0.05$) in the level of serum creatinine in LVA-treated rats (T2 group) compared with

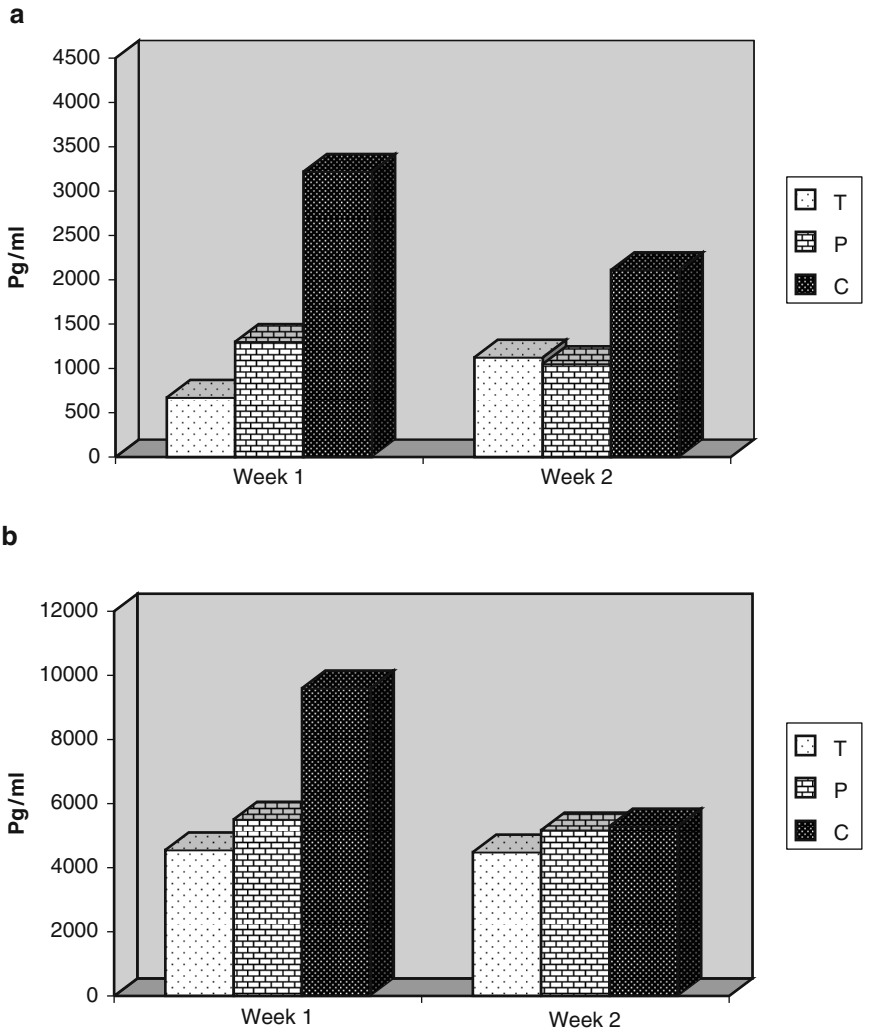

Fig. 8 Amounts of LTB4 (**a**) and PGE2 (**b**) in colonic tissue homogenate following 6 weeks of oral administration of LVA. *C* colitis-induced rats, *P* prophylactic group rats, *T* treated rats (Razavi et al. 2008)

nontreated controls. Moreover, the anti-BSA antibody titers were significantly lower in LVA-treated rats (group T2, 50 mg kg^{-1}) than in nontreated controls at the 12th week after immunization. The changes of the mean levels of anti-BSA antibody titers in the third, sixth, ninth, and 12th experimental weeks are illustrated in Fig. 10.

There was no significant difference in the level of BUN and serum lipids (triglyceride and cholesterol) between different groups. The light microscopy examination of renal tissue revealed the severity of hypercellularity, glomerular infiltration of polymorphonuclear leukocytes (PMN), fibrinoid necrosis, and inter-

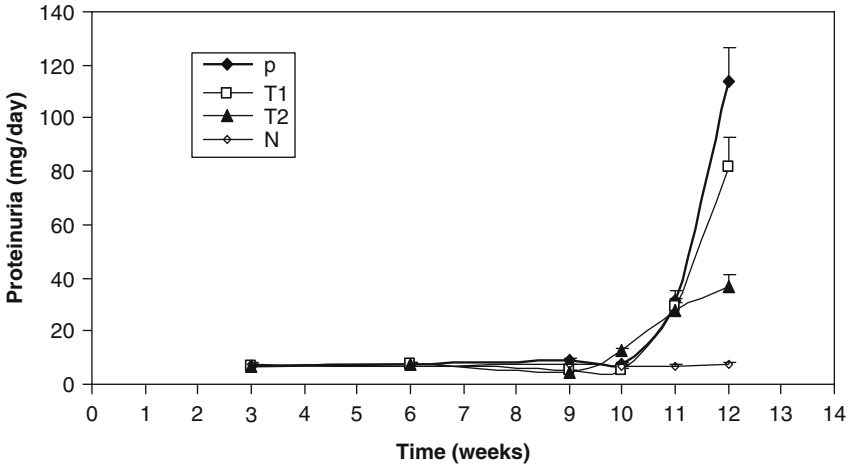

Fig. 9 Time course of the mean values of proteinuria in different groups in immune complex glomerulonephritis: *N* normal rats ($n = 10$), *P* patient rats ($n = 9$), *T1*, *T2* groups treated by intraperitoneal injections of LVA at two different doses, 25 and 50 mg kg^{-1}, respectively ($n = 2 \times 9$). The onset of intraperitoneal administration of LVA to T1 and T2 groups was on day 42. There were 14 intraperitoneal injections, the injection interval was 72 h, and the end of the therapeutic protocol was day 81. There was a significant difference between the patient group and the treated group (T2). $P < 0.05$ was considered significant (Mirshafiey et al. 2005b)

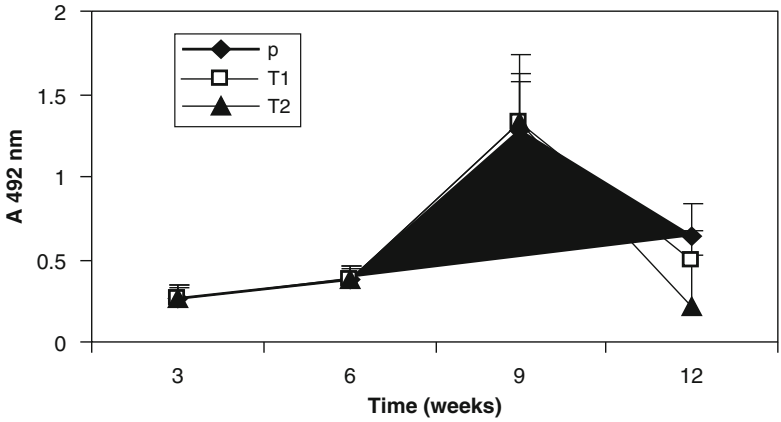

Fig. 10 Comparison of anti-bovine serum albumin (BSA) antibody titers in groups P, T1, and T2 during the course of acute serum sickness. Patient rats were divided into four subgroups ($n = 5 + 5 + 5 + 9$) and treated rats into T1 ($n = 7 + 9$) and T2 ($n = 7 + 9$) subgroups. Rats from patient subgroups were killed in four stages (third, sixth, ninth, and 12th weeks), whereas treated subgroups were killed in two stages (ninth and 12th weeks) after immunization to obtain their sera. Treated groups (T1 and T2) received intraperitoneal injections of LVA at two different doses, 25 and 50 mg kg^{-1}, respectively. The onset of intraperitoneal administration of LVA to T1 and T2 groups was on day 42. As determined by the ELISA method, the highest titers occurred in the P group, the intermediate titers were in the T1 group, and the lowest titers were in the T2 group. At the 12th week, the reduction of anti-BSA antibody in the T2 group was significant compared with that in the P group. Values are means ± S.D (Mirshafiey et al. 2005b)

stitial infiltration in various groups. Rats treated with LVA (group T2) showed a significant reduction in glomerular changes compared with nontreated controls. Immunofluorescence microscopy investigation of glomeruli revealed deposits of immune complexes in the mesangial areas and along the capillary walls of all the rats. Glomerular deposition of immune complex was significantly less intense in LVA-treated rats (group T2) than in nontreated controls. These findings suggest that treatment with sodium alginate as a new immunosuppressive agent can reduce proteinuria, and suppress the antibody production as well as the development of glomerular lesions in a rat model of immune complex glomerulonephritis.

5 Therapeutic Effects of M2000 on an Experimental Model of Rheumatoid Arthritis

This investigation was planned to explore the therapeutic potency of M2000, a novel designed nonsteroidal anti-inflammatory drug in an adjuvant-induced arthritis model (Mirshafiey et al. 2005). For this purpose, a production process for the commercially unavailable M2000 had to be established. An alginate epimerase negative *Pseudomonas* strain was engineered which synthesized homopolymeric polymannuronate. The polymannuronate was subjected to acid hydrolysis to obtain the monomer, which was further purified by gel filtration chromatography. On the basis of the method of (Cuzzocrea et al. 2005), arthritis was induced in Lewis rats by a single intradermal injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* (0.3 mg) in Freund's incomplete adjuvant into the right foot pad. Fourteen days after injection of adjuvant, the contralateral left foot pad volume was measured. The rats with paw volumes 0.37 ml greater than those of normal paws were then randomized into treatment groups. Intraperitoneal administration of test drugs (M2000, 40 mg kg^{-1} per day to group T1 and indomethacin, 2 mg kg^{-1} per day to group T2) and oral administration of M2000 (40 mg kg^{-1} per day to group T3) were started on day 15 after adjuvant injection and continued until the final assessment on day 25. The left hind limb was removed for histological evaluation. The results showed that the oral administration as well as the intraperitoneal injection of M2000 into arthritic rats induced a significant reduction in paw edema. Histopathological assessment showed a reduced inflammatory cell infiltrate in joints of treated rats, and the number of osteoclasts present in the subchondral bone, tissue edema, and bone erosion in the paws were markedly reduced following M2000 therapy.

The data in Fig. 11 demonstrate a time-dependent increase induced by adjuvant in the left hind paw volume (milliliters) of rats. The intraperitoneal injection of M2000 into arthritic rats could rapidly reverse paw edema as did indomethacin, and after 10 days of treatment paw swelling was significantly ($P < 0.05$) reduced in M2000-treated rats compared with vehicle-treated controls. On the other hand, as is apparent in Fig. 12, oral administration of M2000 (40 mg kg^{-1}) in arthritic rats induced a significant reduction in paw edema. After 10 days of treatment, paw swelling was reduced in M2000-treated rats relative to the paw volume of vehicle-treated rats. This difference was statistically significant. There was no macroscopic evidence of hind paw erythema and/or edema in the normal control rats.

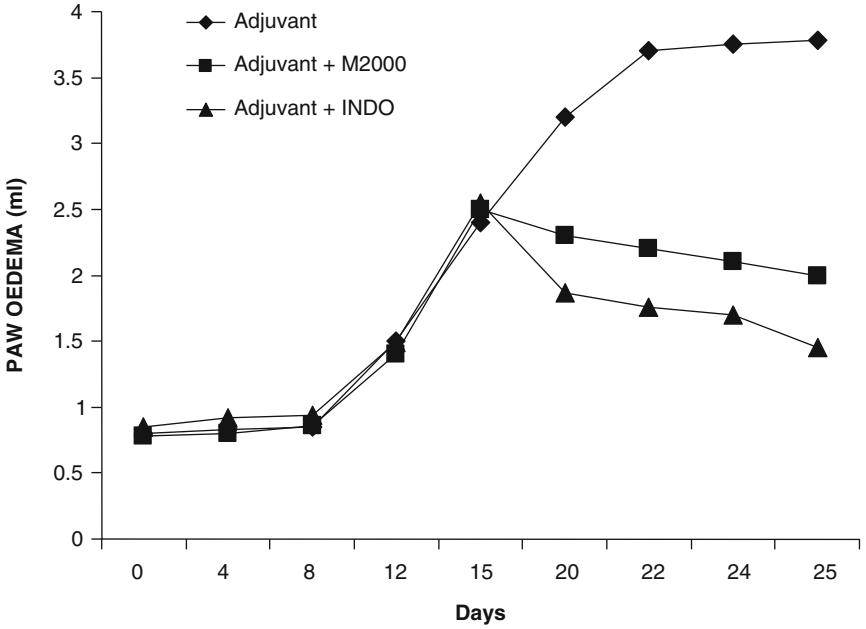

Fig. 11 Severity of arthritis score in treated groups (A + M) compared with nontreated control rats (**a**). A time-dependent increase induced by adjuvant in the left hind paw volume (milliliters) of rats is demonstrated. The intraperitoneal injection of β-D-mannuronic acid (M2000) to arthritic rats could rapidly reverse paw edema as did indomethacin, and after 10 days of treatment paw swelling was significantly ($P < 0.05$) reduced in M2000-treated animals compared with vehicle-treated controls (Mirshafiey et al. 2005c)

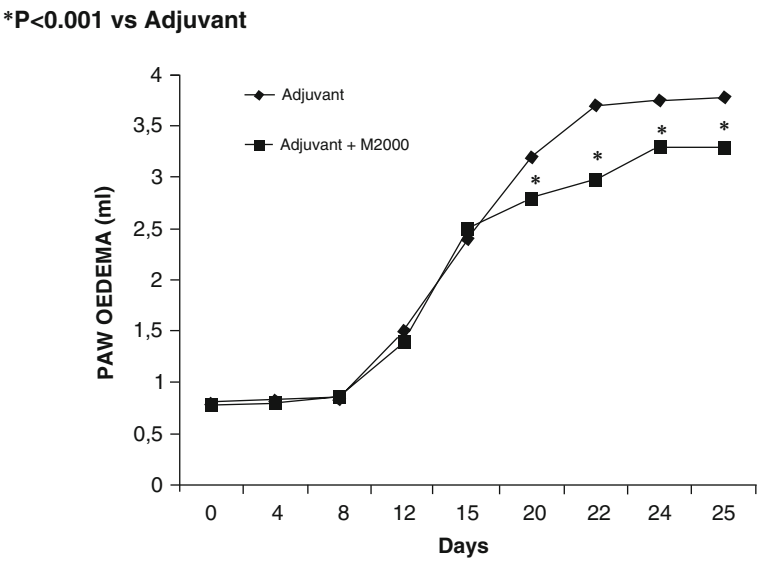

Fig. 12 The oral administration of M2000 (40 mg kg^{-1}) in arthritic rats induced a significant ($P < 0.05$) reduction in paw edema. After 10 days of treatment paw swelling was reduced in M2000-treated animals relative to the paw volume of vehicle-treated rats. This difference was statistically significant, $P < 0.05$ (Mirshafiey et al. 2005c)

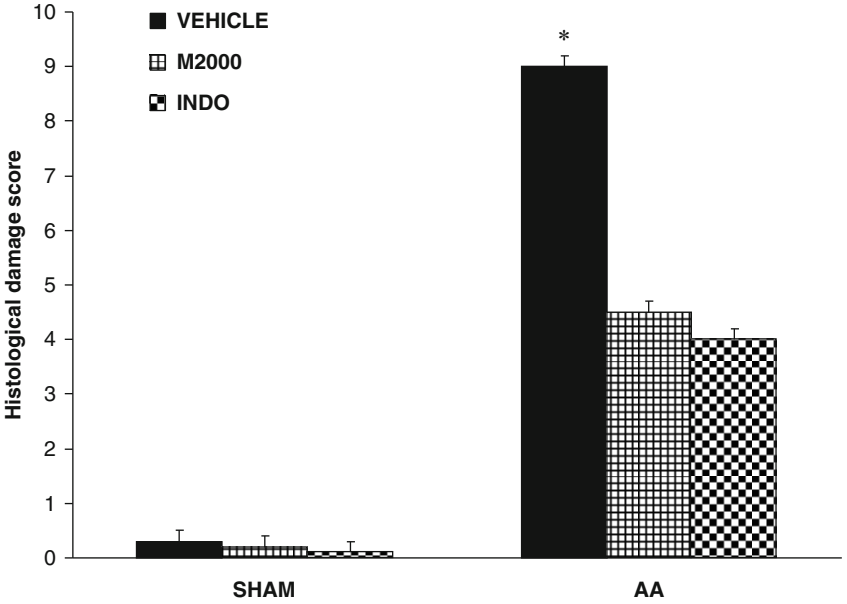

Fig. 13 Effect of M2000 treatment on histological damage score. The histological evaluation of the paws in the vehicle-treated arthritic rats reveals signs of severe arthritis along with inflammatory cell infiltrate. M2000 therapy could significantly reduce the pathological parameter compared with that of the control group. $P < 0.05$ was considered significant (Mirshafiey et al. 2005c)

As shown in Figs. 13 and 14, histological evaluation of the paws in the vehicle-treated arthritic rats reveals signs of severe arthritis along with inflammatory cell infiltrate. Histopathological assessment showed a reduced inflammatory cell infiltrate in the joints of treated rats, and the number of osteoclasts present in the subchondral bone, tissue edema, and bone erosion in the paws were markedly reduced by both treatments, indicating that the drugs tested were effective in retarding synovial inflammation and prevented joint destruction. Treatment with M2000 and indomethacin resulted in preservation of hyaline in the articular cartilage. Moreover, subchondral bone was intact and the numbers of osteoclasts in the subchondral and trabecular bone space were greatly reduced.

Taken together; the data obtained show that M2000, as a novel nonsteroidal anti-inflammatory drug, could be suggested as an anti-inflammatory drug for long-term administration.

6 Immunosuppressive Effect of M2000 in Experimental Multiple Sclerosis

The therapeutic potency of M2000 as a novel designed nonsteroidal anti-inflammatory drug with immunosuppressive property in a T-cell-mediated autoimmune disease was tested (Mirshafiey et al. 2005). The influence of M2000 on experimental autoimmune

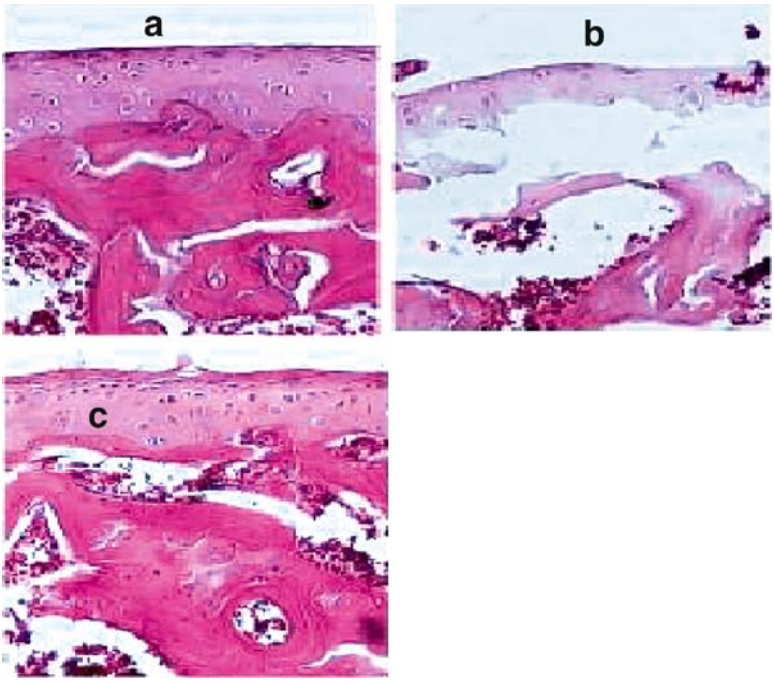

Fig. 14 Representative histopathological slides of a hind limb joint of a healthy Lewis rat (**a**), a rat with adjuvant-induced arthritis (**b**), and an arthritic rat treated with M2000 (**c**). The joint of the rat which had been treated with M2000 (**c**) shows significantly fewer signs of joint destruction (Mirshafiey et al. 2005c)

encephalomyelitis (EAE), an animal model of multiple sclerosis, induced by myelin basic protein (MBP) was assessed. M2000 at two doses, 40 and 80 mg kg^{-1} per day, was administered intraperitoneally to prevention and treatment groups, respectively. The onset of intraperitoneal injections of M2000 for prophylactic and therapeutic groups was on the first and seventh days after immunization. Rats were divided at random into a prevention group with two subgroups: M1 – M2000-pretreated rats, which received in total 18 intraperitoneal injections from 1 day before immunization (day 1) to day 16 after immunization; C1 – control patient rats, which received intraperitoneal injections of saline during the same period (from day 1 to day 16). The treatment group included two subgroups: M2 – M2000-treated rats, which received in total 14 intraperitoneal injections from day 7 to day 20 after immunization; C2 – control patient rats, which received intraperitoneal injections of saline during the same period. Rats were killed on day 21 after immunization. The results of this experiment showed that the treatment of EAE with M2000 could significantly suppress disease development both prophylactically and therapeutically; the onset and symptoms of EAE in Lewis rats could be suppressed following the administration of M2000. Clinical improvement was accompanied by a marked decrease in the mean numbers of vessels with perivascular cellular

infiltration in M2000-treated rats compared with nontreated control. Disease suppression was associated with a marked suppression of MBP-specific T-cell reactivity in vitro, without any evidence for a generalized impairment of T-cell activity.

Following the chronological changes of the clinical score of each group of the EAE rats (Figs. 15, 16), the first clinical signs appeared in some rats of each group on day 10 and then the symptoms reached their maximum level on day 13 after immunization. The maximum severity score of EAE was 5 in each group. The rats pretreated and treated with M2000 were less affected than the controls (Figs. 15 and 16). Three rats from the M1 subgroup did not develop apparent signs of EAE during the period of observation, whereas all of the rats from the C1 and C2 subgroups developed typical signs (clinical score more than 4) of EAE. As shown in Figs. 15 and 16, the mean clinical scores of rats for subgroups M1 and M2 were significantly less than those of control rats (the two-way repeated measures analysis of variance, $P < 0.05$). The mortality rate in the M1 subgroup (40 mg kg^{-1} per day) was 0/7 compared with 0/8 for the C1 subgroup, whereas, interestingly, the mortality rate in the M2 subgroup (80 mg kg^{-1} per day) was 0/6 compared with 2/8 in the C2 subgroup. The mean body weight more reduced in controls than in subgroup M1 rats after day 12 (Fig. 17). Moreover, lymph node cells from the rats treated with M2000 exhibited a reduced proliferative response to MBP in contrast to those of control patient rats, whereas the proliferative responses of lymph node cells to Con A were almost the same in both groups (Fig. 18). On the other hand, the histopathological assessment of brain and spinal cord sections in treated and nontreated groups showed that the mean numbers of vessels with perivascular cellular infiltration in brain (0.57 ± 0.79) and spinal cord (2.0 ± 2.65) in M2000-treated rats were

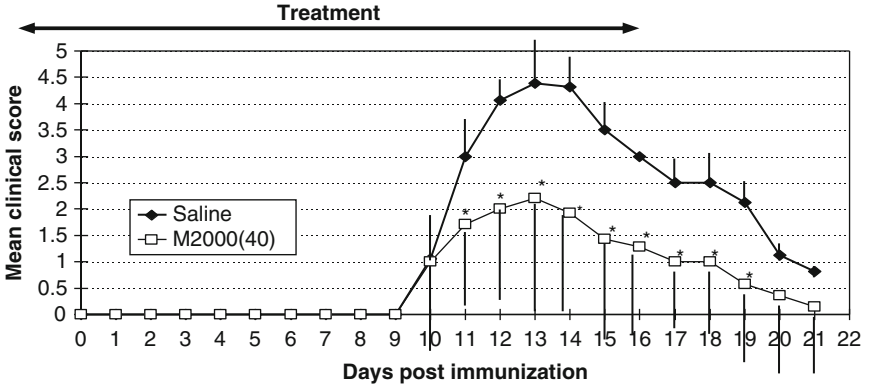

Fig. 15 Amelioration of myelin basic protein (MBP) induced experimental autoimmune encephalomyelitis (EAE) by preventive application of M2000. Fifteen Lewis rats were immunized with 50 μg MBP in complete Freund's adjuvant into one hind footpad and were then divided into two subgroups. M2000-pretreated rats ($n = 7$) received in total 18 intraperitoneal injections of M2000 (40 mg kg^{-1} per day) from 1 day before immunization to day 16 after immunization. Control rats ($n = 8$) received saline intraperitoneal injections during the same period. M2000 therapy caused a significant reduction in clinical score compared with that for nontreated controls ($P < 0.05$). *Bars* indicate ± SD (Mirshafiey et al. 2005d)

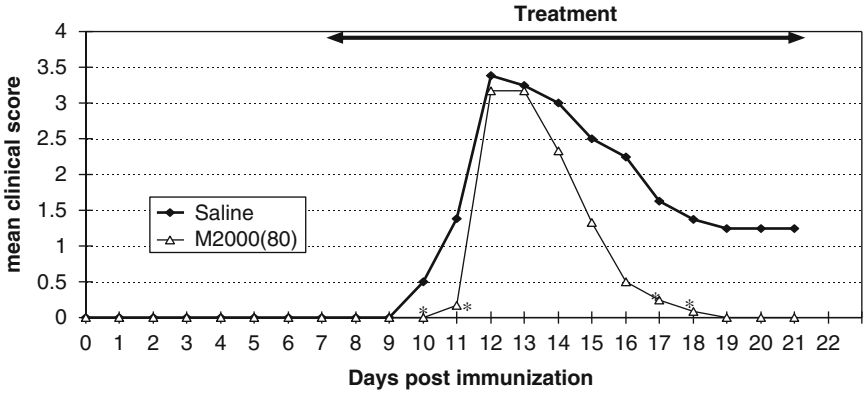

Fig. 16 Amelioration of MBP-induced EAE by therapeutic application of M2000. Lewis rats were immunized with 50 μg MBP in CFA into one hind footpad and were then divided into two subgroups. M2000-treated rats ($n = 6$) received in total 14 intraperitoneal injections of M2000 (80 mg kg^{-1} per day) from day 7 to day 20 after immunization. Control rats ($n = 8$) received intraperitoneal injections of saline during the same period. M2000 therapy caused a significant reduction in clinical score compared with that for nontreated control ($P < 0.05$) (Mirshafiey et al. 2005d)

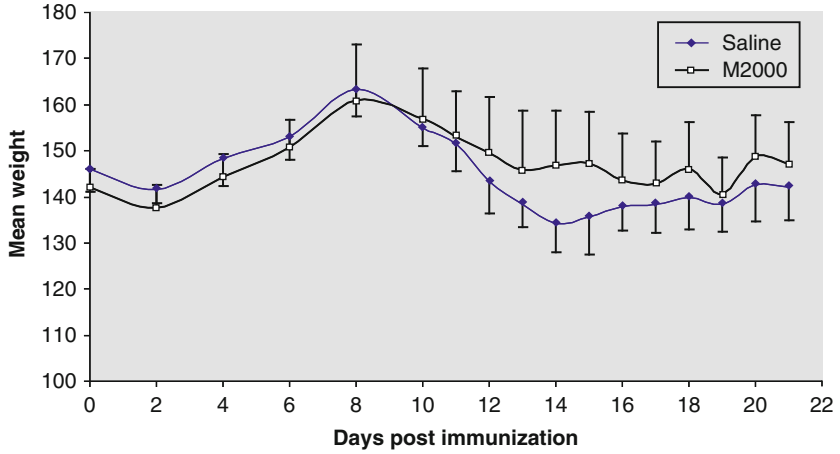

Fig. 17 The higher weight gain in M2000-treated rats compared with nontreated controls during the EAE period. M2000-treated rats ($n = 7$) received in total 18 intraperitoneal injections of M2000 (40 mg kg^{-1} per day) from 1 day before immunization to day 16 after immunization. Control rats ($n = 8$) received intraperitoneal injections of saline during the same period. *Bars* indicate SD (Mirshafiey et al. 2005d)

significantly less than in nontreated controls, with mean values of 2.43 ± 3.41 for brain and 10.71 ± 8.76 for spinal cord, respectively.

Collectively, our data suggest that M2000 may provide a novel therapeutic option for T-cell-mediated autoimmune diseases in humans.

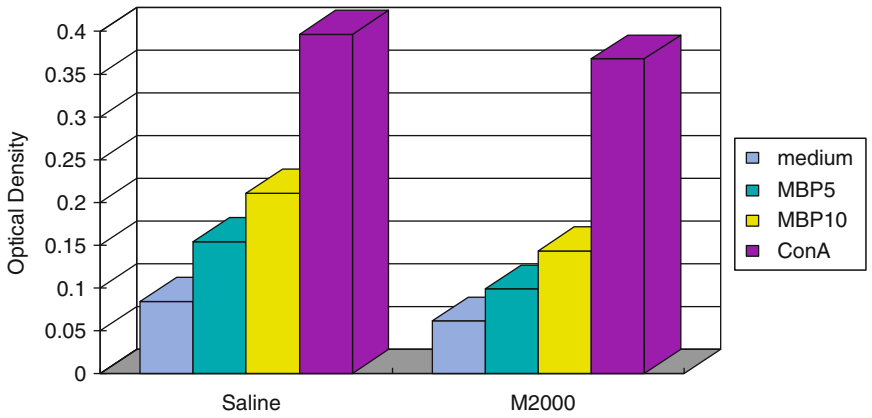

Fig. 18 The effect of M2000 at two different doses (5 and 10 μg ml^{-1}) on proliferative responses of regional lymph node cells from EAE rats, "$n = 4$, for each group, on day 21 after immunization" (Mirshafiey et al. 2005d)

7 Treatment of Experimental Nephrotic Syndrome with M2000

The therapeutic effect of the M2000 molecule was tested in adriamycin-induced nephropathy (Mirshafiey et al. 2004a). To induce the experimental nephrotic syndrome, adriamycin was given once by a single intravenous injection (7.5 mg kg^{-1}) through the tail vein. Six days after injection of adriamycin, a therapeutic protocol was developed by intraperitoneal administration of 30 mg kg^{-1} M2000 solution to the treatment 1 (T1) group and intraperitoneal injection of piroxicam (0.3 mg kg^{-1}) for the treatment 2 (T2) group. In total there were 14 intraperitoneal injections, of which five injections were performed day after day and nine injections were carried out at regular 48-h intervals. The therapeutic protocol was terminated on day 28 and the rats were killed on day 43. The treated patient rats showed a significant reduction in proteinuria, BUN, serum creatinine, and serum cholesterol, and administration of M2000 could significantly diminish the serum level of IL-6 in treated rats compared with nontreated controls. Moreover, treatment with M2000 significantly reduced the number of glomerular leukocytes, hypercellularity, and hydropic change in the capillary network within the renal cortex and decreased tubular casts.

The changes of the mean levels of urinary protein excretion between normal, patient, T1, and T2 groups are shown in Fig. 19. This experiment showed that intraperitoneal administration of M2000 (30 mg kg^{-1}) could exert its therapeutic effects on adriamycin-induced nephropathy. For exact evaluation of the effects of M2000, the experiment was terminated 14 days after the last injection of M2000. The urinary protein excretion was significantly less in M2000-treated rats than in nontreated controls and piroxicam-treated rats.

Figure 20 shows the antiproteinuric effect of M2000 therapy compared with that of piroxicam at the end of experiment (day 42). Here there was significant difference

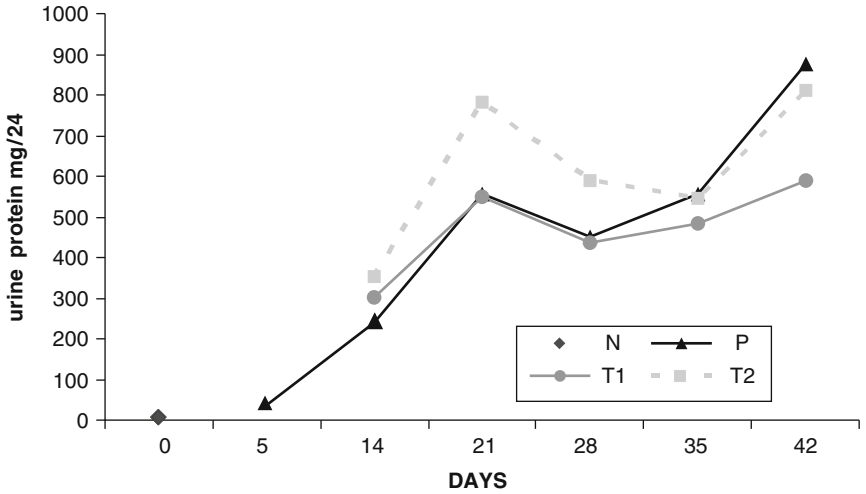

Fig. 19 Time course of the mean values of proteinuria in different groups in experimental nephrosis: *N* normal rats ($n = 8$), *P* patient (nontreated) rats that show an increase of proteinuria levels after day 5 ($n = 9$), *T1*, *T2* groups treated by intraperitoneal injections of M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively ($n = 2 \times 8$). Note: a single intravenous injection of adriamycin (7.5 mg kg^{-1} body weight) induced a severe nephrotic syndrome. The onset of intraperitoneal administration of M2000 and piroxicam to groups T1 and T2 was on day 6 (after development of disease).There were significant differences between nontreated (P) and M2000-treated rats (T1). $P < 0.05$ was considered significant (Mirshafiey et al. 2004a)

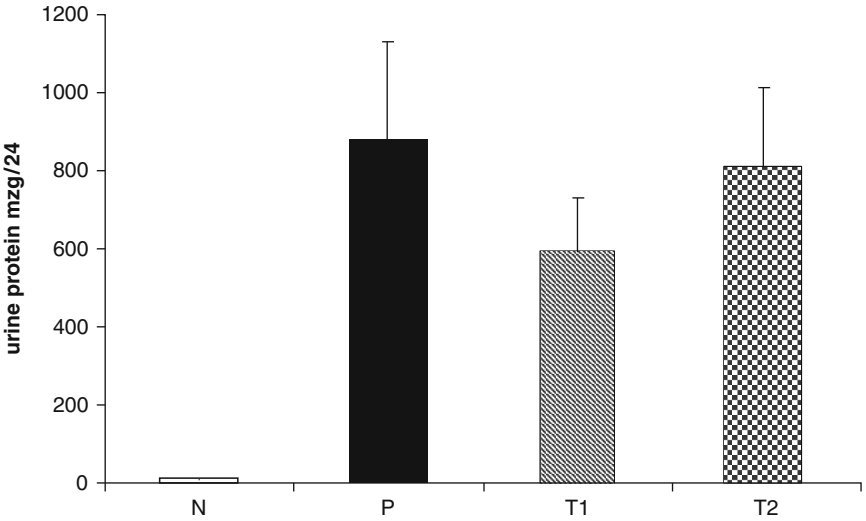

Fig. 20 Effect of M2000 on proteinuria at the end of the experiment (day 42) in different groups: *N* normal rats (8), *P* patients rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. The comparison of the antiproteinuric effect of M2000 between groups P and T1 showed a significant difference, "$P < 0.05$ (Mirshafiey et al. 2004a)

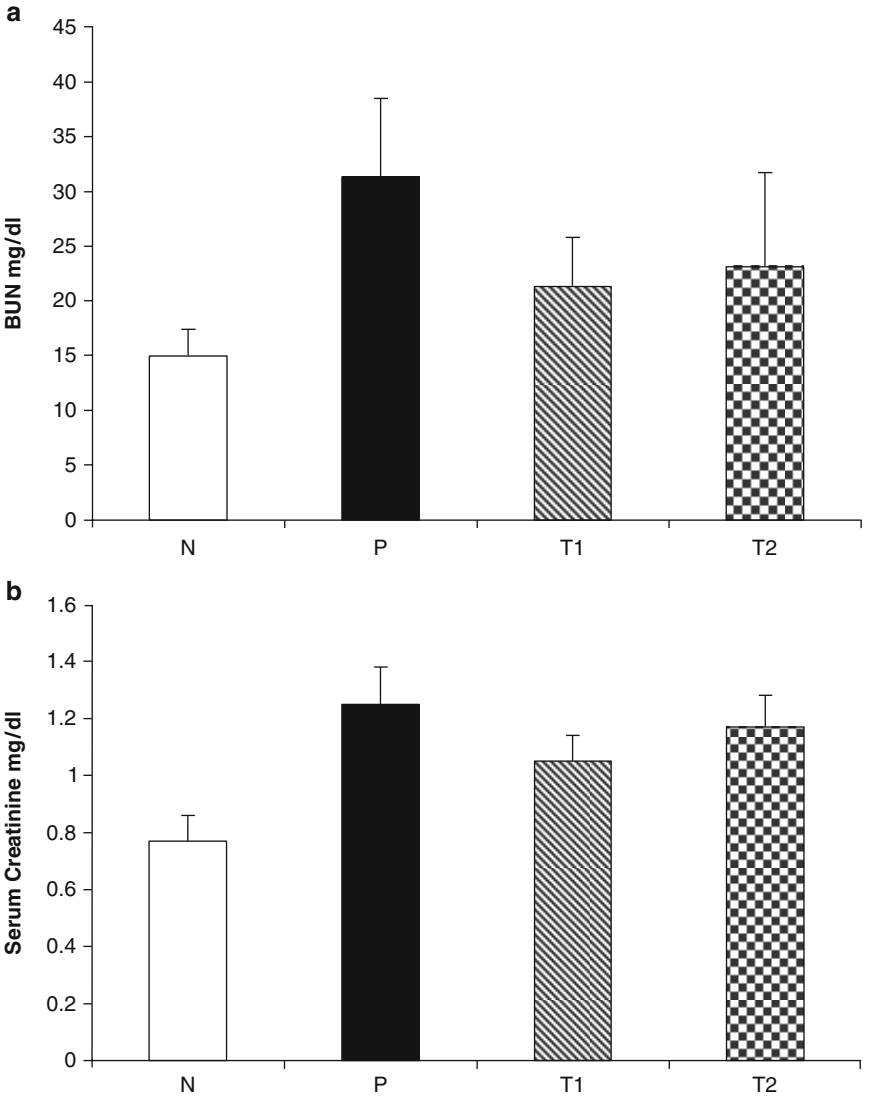

Fig. 21 Effect of M2000 on serum blood urea nitrogen and serum creatinine in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. **a** BUN concentration in groups N, P, T1, and T2. BUN concentration in group T1 vs. group P was significant, $P < 0.05$. **b** Creatinine concentration in groups N, P, T1, and T2. Creatinine concentration in group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2004a)

between T1 and patient groups. In Fig. 21a, the amounts of BUN are compared between the different groups (normal, patient, T1, and T2). This figure shows that the difference between treated rats (T1 group) and nontreated rats (patient group)

is significant. In Fig. 21b, the comparison of serum creatinine concentration among various groups shows that there is a significant difference between T1 and patient groups, whereas there were no significant differences in the levels of urinary urea nitrogen and urine creatinine concentration between M2000-treated rats and nontreated controls (data not shown).

In addition, serum cholesterol and triglyceride levels were significantly elevated in nephrotic rats when compared with the healthy controls at the end of the experiment. Intraperitoneal injections of M2000 solution into patient rats significantly reduced serum cholesterol levels in nephrotic rats (Fig. 22).

Figure 23 shows the effect of M2000 therapy on the reduction of IL-6 production in treated patient rats (group T1). The difference between T1 and patient groups in terms of IL-6 concentration was significant.

Light microscopy examination of renal tissue revealed the severity of hypercellularity, glomerular infiltration of PMN, hydropic change in the capillary network within the renal cortex, and the existence of tubular casts in the various groups. The rats treated with M2000 showed a significant reduction in glomerular changes compared with nontreated controls (Table 1).

These data suggest that M2000 therapy can ameliorate proteinuria and suppress the progression of glomerular lesions in an experimental model of nephrosis.

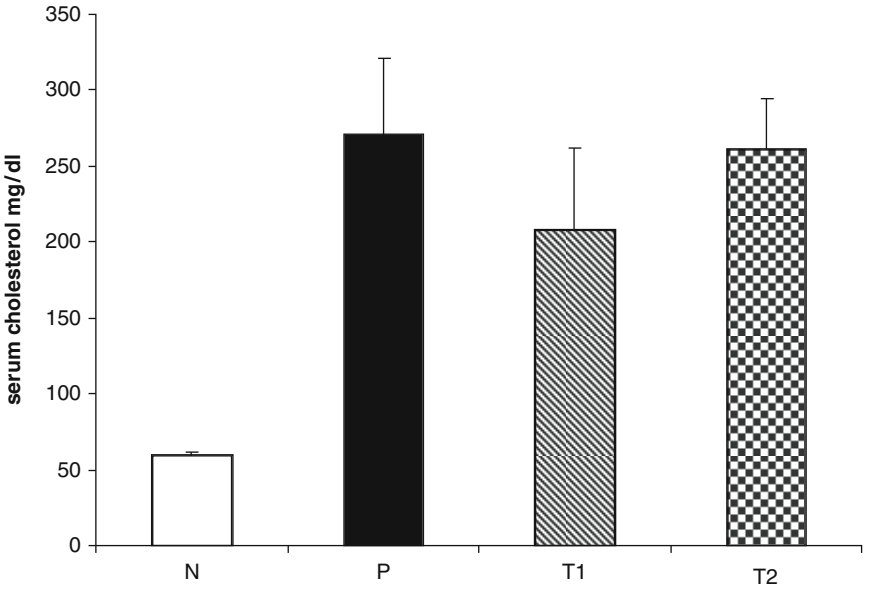

Fig. 22 Effect of M2000 on serum cholesterol level in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. Cholesterol concentration in group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2004a)

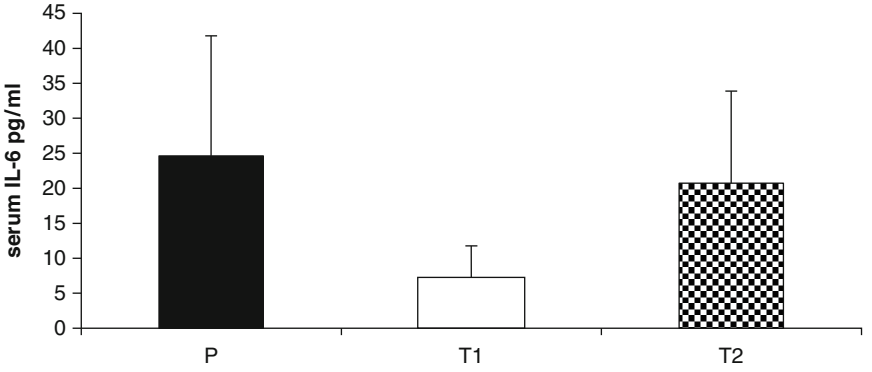

Fig. 23 Effect of M2000 on serum IL-6 level in different groups: *N* normal rats (8), *P* patient rats (9), *T1* rats treated with M2000 (8), *T2* rats treated with piroxicam (8). Each *bar* represents the mean ± SD. The Difference between group T1 and group P in terms of IL-6 concentration was significant, $P < 0.05$, (Mirshafiey et al. 2004a)

t.1 **Table 1** Light microscopy findings of kidney histological lesions in various groups

t.2 t.3 Group	No. of rats	Hypercellularity	Polymorphonuclear leukocyte infiltration	Cast	Hydropic change
t.4 N	8	0.40 ± 0.53	–	–	–
t.5 P	9	1 ± 0.57	1.71 ± 0.48	2.14 ± 0.69	1 ± 0
t.6 T1	8	0.57 ± 0.53	1	1.14 ± 0.63	–
t.7 T2	8	0.71 ± 0.46	1.6 ± 0.76	1.87 ± 1.1	–
t.8 H	8	0.42 ± 0.53	–	–	–

t.9 Values are expressed as the mean ± the standard deviation. Semiquantitative scoring of histological
t.10 lesions shows the significant differences between groups P and T1, in hypercellularity, in glomerular
t.11 polymorphonuclear leukocyte infiltration, hydropic change in the capillary network within the renal
t.12 cortex, and in tubular casts, ($P < 0.05$). Group H was the healthy control receiving β-D-mannuronic
t.13 acid (M2000; 30 mg kg^{-1})

8 Therapeutic Effects of M2000 in Experimental Immune Complex Glomerulonephritis

The therapeutic efficacy of the novel anti-inflammatory agent M2000 in an experimental model of immune complex glomerulonephritis was evaluated (Mirshafiey et al. 2007). BSA nephritis as an experimental model of glomerulonephritis was induced in rats by a subcutaneous immunization and daily intravenous administration of BSA. Rats were divided randomly into five groups. normal; patient; patient groups treated with M2000 and piroxicam (T1 and T2, respectively); a healthy control receiving M2000. The M2000 solution (30 mg kg^{-1}) was administered intraperitoneally at regular 48-h intervals for 4 weeks. The onset of treatment was on day 56. Urinary protein was measured weekly and serum anti-BSA antibody was assessed by ELISA at different intervals. The rats were killed on day 84 and blood samples and

kidney specimens were obtained. Serum (creatinine, BUN, cholesterol, and triglyceride) and urine (protein, urea, and creatinine) determinants were measured at the time of death. Kidney specimens were processed for light and immunofluorescence microscopy examination. The results showed that M2000 therapy could significantly reduce the urinary protein excretion in treated rats compared with nontreated controls. The anti-BSA antibody titer was lower in treated rats than in controls at the 12th experimental week. There were no significant differences in the levels of serum determinants, urine urea, and creatinine between control and treated rats. PMN infiltration and glomerular immune complex deposition were less intense in treated rats than in controls. Figure 24 shows the changes of the mean levels of urinary protein excretion between various groups; normal, patient, T1, and T2. The urinary protein excretion was significantly less in M2000-treated rats than in nontreated controls.

Figure 25 shows the antiproteinuric effect of M2000 therapy compared with patient and piroxicam (T2) groups at the end of the experiment (day 84). Here, the reduction of proteinuria in T1 compared with patient and T2 groups was significant.

Figure 26 illustrates the changes of the mean levels of anti-BSA antibody titers in the sixth, ninth, and 12th experimental weeks. The anti-BSA antibody titers were significantly lower in M2000-treated rats (T1 group) than in piroxicam-treated rats (T2 group) and nontreated controls (patient group) at the end of the experiment (day 84).

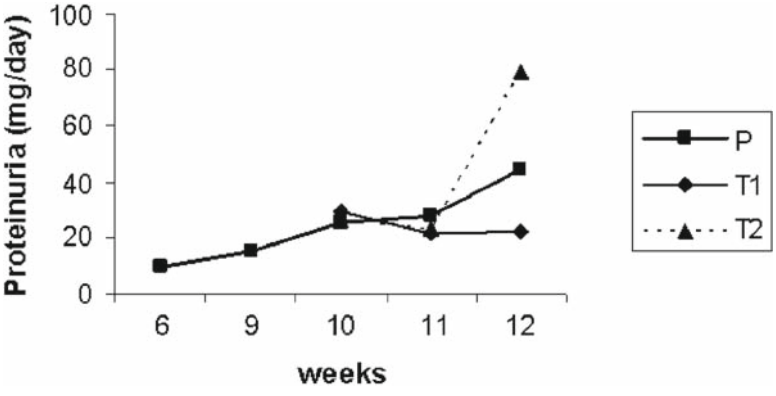

Fig. 24 Time course of the mean values of proteinuria in different groups in immune complex glomerulonephritis: *N* normal rats ($n = 8$), *P* patient rats ($n = 9$), *T1*, *T2* rats treated with intraperitoneal injections of M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively ($n = 2 \times 8$). The onset of intraperitoneal administration of M2000 and piroxicam to T1 and T2 groups was on day 56. There were 15 intraperitoneal injections, the injection interval was 48 h, and the end of the therapeutic protocol was day 84. There was a significant difference between the M2000-treated group (T1) and the patient group. $P < 0.05$ was considered significant (Mirshafiey et al. 2007)

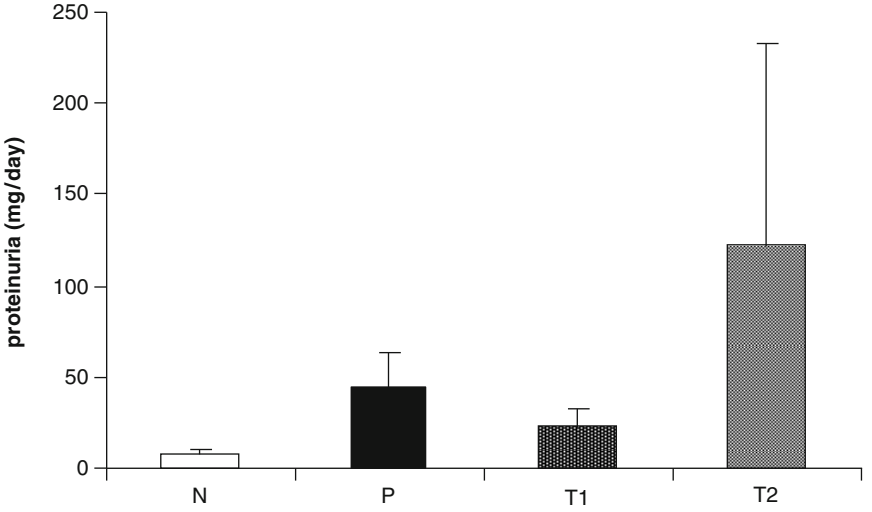

Fig. 25 Comparison of the antiproteinuric effect of M2000 between different groups at the end of experiment (week 12). *N* normal rats (8), *P* patient rats (9), *T1*, *T2* rats treated with M2000 (30 mg kg^{-1}) and piroxicam (0.3 mg kg^{-1}), respectively (2 × 8 = 16). Each *bar* represents the mean ± S.D. The antiproteinuric effect for group T1 vs. group P was significant, $P < 0.05$ (Mirshafiey et al. 2007)

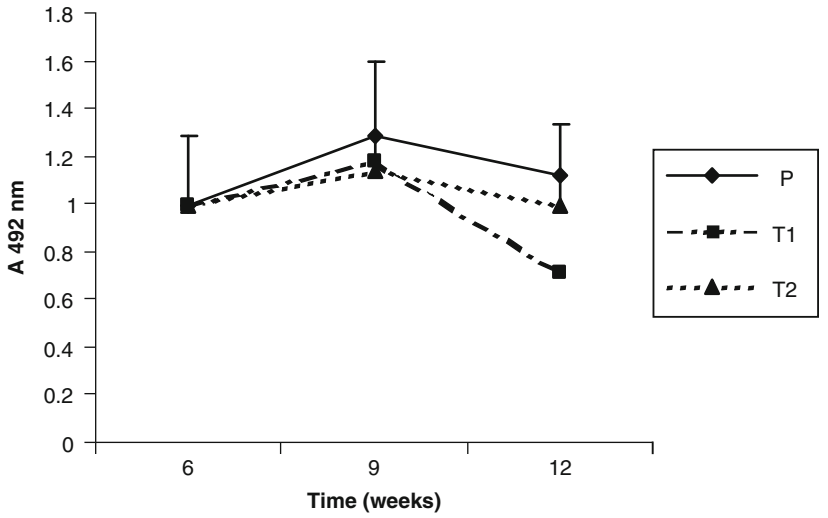

Fig. 26 Anti-BSA antibody titers in groups P, T1, and T2 during the course of acute BSA nephritis, as determined by an ELISA method. The highest titer occurs in group P, the intermediate in group T2, and the lowest in group T1. Values are means ± S.D. The difference between the M2000-treated group (T1) and the patient group (P) at the 12th week was significant. $P < 0.05$ was considered significant (Mirshafiey et al. 2007)

Light microscopy examination of renal tissue revealed the severity of glomerular infiltration of PMN in nontreated rats (patient group) and in rats treated with piroxicam (T2 group) compared with M2000-treated rats (T1 group) (data not shown). Immunofluorescence microscopy investigation of glomeruli revealed that glomerular immune complex deposition was less intense in rats treated with M2000 (T1 group) than in controls (patient group) and in rats treated with piroxicam (data not shown).

These findings suggested that treatment with M2000 ($C_6H_{10}O_7$) can reduce proteinuria, diminish antibody production, and suppress the progression of disease in a rat model of immune complex glomerulonephritis.

9 Tolerability and Pharmacotoxicology of M2000

The pharmacotoxicology study of M2000 was carried out on animal models based on the evaluation of serum and urine determinants, structure of kidney, gastrointestinal tolerability, and body temperature (Mirshafiey et al. 2004). Moreover, the WEHI-164 (fibrosarcoma) cell line was used to assay tolerability and matrix metalloproteinase type 2 (MMP-2) activity, on the basis of the method of (Heussen and Dowdle 1980). MMP-2 activity was assessed using zymography.

Toxicology studies were categorized on the basis of:

1. Serum and urine determinants: To evaluate the side effects of M2000, assessment of kidney function was performed in healthy rats receiving the test drug after 12 intraperitoneal injections of M2000 (30 mg kg^{-1} each 48 h) and was based on the measurements of serum creatinine, BUN, urinary protein excretion, urine urea, as well as the plasma concentration of triglyceride and cholesterol.
2. Histological examinations: Renal tissues were assessed using light microscopy. Glomerular lesions were graded on a scale of 0–3 (0, *negative*; 1, *mild*; 2, *moderate*; 3, *marked*) according to four parameters: hypercellularity, glomerular infiltration of PMN, hydropic change in the capillary network within the renal cortex, and the presence of tubular casts.
3. Gastroulcerogenic study: After 12 intraperitoneal injections of M2000 (30 mg^{-1} kg^{-1} each 48 h) for the group of healthy control rats, as well as oral administration of M2000 (40 $mg^{-1}kg^{-1}$ per day) for 10 days to group T3, the rats were killed and the stomach and the duodenum were dissected out. Scoring was done on the basis of at least one gastric ulcer or one hemorrhagic erosion.
4. Body temperature: The course of rectal temperature of rats which received 12 intraperitoneal injections of M2000 solution (30 mg^{-1} kg^{-1} each 48) was monitored continuously by thermoelements and compared with the baseline temperature in normothermic controls.

Cytotoxicity analysis showed a much higher tolerability for M2000 than for other drugs tested (diclofenac, piroxicam, and dexamethasone). The inhibitory effect of M2000 on MMP-2 activity was significantly greater than that of dexsamethasone

and of piroxicam at a concentration of 200 μg ml^{-1}. Moreover, data analysis showed no significant differences in the levels of serum determinants (BUN, creatinine, cholesterol, and triglyceride) and urine determinants (protein excretion and creatinine) between the normal group and the healthy experimental group challenged with M2000 (Table 2), whereas a significant increase of urine urea in the healthy group compared with the normal group revealed the advantage using M2000 in healthy controls (Table 2). The histological study of kidney specimens obtained from the normal group and healthy controls receiving M2000 showed no glomerular changes

Table 2 Toxicological inspection of M2000 administration on serum and urine determinants

Group	No. of rats	Blood urea nitrogen (mg dl^{-1})	Serum creatinine (mg dl^{-1})	Serum cholesterol (mg dl^{-1})	Serum triglyceride (mg dl^{-1})	Urine protein (mg day^{-1})	Urine urea (mg dl^{-1})
N	8	16 ± 2	0.7 ± 0.1	59 ± 3	79 ± 9	8 ± 3	939 ± 300
H	8	17 ± 4	0.8 ± 0.1	56 ± 6	89 ± 23	6 ± 2	1,284 ± 226

The healthy group receiving M2000 (*H*) versus the normal (*N*) group shows no significant difference in serum and urine determinants.

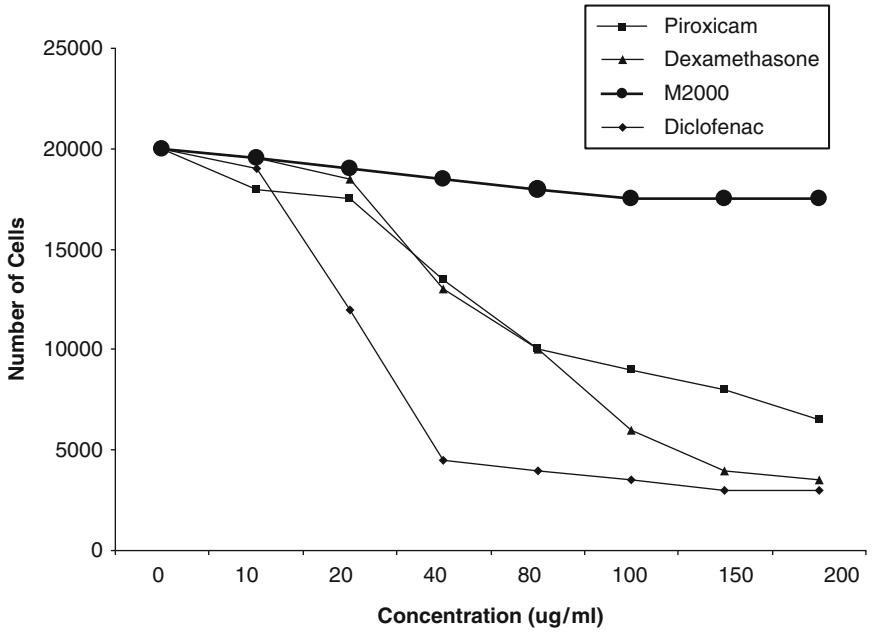

Fig. 27 Cytotoxic effect of M2000. Proliferative response of fibrosarcoma (WEHI-164) cell line to M2000 at different doses (10–200 μg ml^{-1}) compared with those of diclofenac, piroxicam, and dexamethasone. LD_{50} for diclofenac, piroxicam, and dexamethasone was 25, 80, and 80 μg ml^{-1}, respectively. In contrast, WEHI-164 as a sensitive cell line showedhigh tolerability against increasing amounts of M2000 (Mirshafiey et al. 2004b)

in the healthy group in comparison with the normal group (data not shown). On the other hand, administration of M2000 had no influence on body temperature of normothermic rats which had received the test drug. Additionally, the results of this experiment showed that 12 intraperitoneal injections of M2000 solution (30 mg kg^{-1} each 48 h) as well as oral administration of M2000 (40 mg kg^{-1} per day) for 10 days could not provoke gastromucosal lesions in the rats of the experimental group.

Figure 27 shows the proliferative response of a fibrosarcoma (WEHI-164) cell line to M2000 at different doses (10–200 μg ml^{-1}) compared with the proliferative responses to diclofenac, piroxicam, and dexamethasone. The tolerability and biocompatibility of WEHI-164, as a sensitive cell line against increasing amounts of M2000, was very high, whereas 50% of cells died when diclofenac, dexamethasone, and piroxicam were added to tissue culture at doses of 25, 80, and 80 μg ml^{-1}, respectively. M2000 showed no cytotoxic effect compared with steroidal and nonsteroidal drugs tested. Figure 28 presents the dose response analysis of the effect of M2000 on MMP-2 activity compared with the effect of various drugs. The inhibitory effect of M2000 at

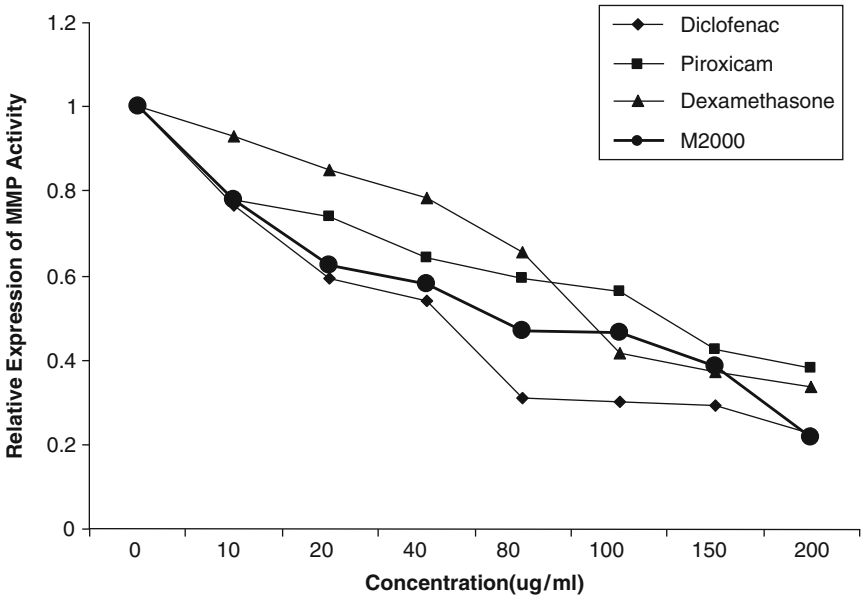

Fig. 28 The inhibitory effect of M2000 on matrix metalloproteinase type 2 activity. Fibrosarcoma cell lines (2×10^4 cell $wall^{-1}$) were incubated overnight with increasing doses of M2000. Diclofenac-, piroxicam- and dexamethasone-treated cells were used as controls. M2000-treated and nontreated cells were investigated in triplicate. The inhibitory activity of M2000 paralleledthat of diclofenac at doses of 10, 20, 40, and 200 μg ml^{-1}, whereas the inhibitory effect of M2000 at concentrations of 20, 40, and 80 μg ml^{-1} was significantly more than that of dexamethasone ($P < 0.05$). Moreover, the inhibitory activity of this novel agent at a dose of 200 μg ml^{-1} was significantly more than that of dexamethasone and that of piroxicam, $P < 0.05$ (Mirshafiey et al. 2004b)

concentrations of 20, 40, 80, and 200 μg ml^{-1} was significantly more than that of dexamethasone ($P < 0.05$), and this difference was significant between M2000 and piroxicam at a concentration of 200 μg ml^{-1}, ($P < 0.05$).In contrast, the inhibitory activity of this novel agent paralleled that of diclofenac at doses of 10, 20, 40, and 200 μg ml^{-1}.

10 Outlook

Alginates have been considered for medical applications such as drug delivery, wound coverage material, and cell encapsulation/transplantation. However, only recently have alginate and its comonomer M2000 been described as potential drugs suitable, in particular, for the treatment of inflammatory diseases. As compiled in this chapter, alginate as well as its comonomer M2000 show efficacy as anti-inflammatory drugs when tested in various inflammatory diseases. The direct application of alginate/M2000 as a medical drug requires the production of alginates with consistent composition and high purity as well as the production of tailor-made alginates such as homopolymeric polymannuronate. Hence, the use of (engineered) microorganisms as production organisms is becoming increasingly attractive and fermentative production might in future be the only feasible production process.

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