© Med Sci Monit, 2005; 11(8): PI53-63
PMID: 16049391

Received: 2004.10.26
Accepted: 2005.03.31
Published: 2005.08.01

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

M2000: a revolution in pharmacology

Abbas Mirshafiey[1ABDEF], Salvatore Cuzzocrea[2BCD], Bernd H.A. Rehm[3BCD], Hidenori Matsuo[4BCD]

[1] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2] Institute of pharmacology, School of Medicine, University of Messina, Messina, Italy
[3] Institut fur Mikrobiologie, Westfalische Wilhelms-Universitat Munster, Munster, Germany
[4] Department of Neurology and Institute of Clinical Research, Nagasaki Medical Center for Neurology, Nagasaki, Japan

Source of support: M2000 has been awarded its international patent cooperation treaty (WO 2004033472) by funding by the University of Munster in Germany

PI

Summary

Background: The tolerability and the anti-inflammatory and immunosuppressive properties of a novel designed non-steroidal anti-inflammatory drug, M2000 (β-D-mannuronic acid), were investigated in various experimental models.

Material/Methods: The anti-inflammatory and immunosuppressive properties of M2000 were tested in experimental models of rheumatoid arthritis (AIA) and multiple sclerosis (EAE). Its therapeutic potency on kidney diseases was studied using experimental models of nephrosis and immune complex glomerulonephritis (ICG). Biocompatibility and pharmacotoxicology assessment of M2000 was carried out using a fibrosarcoma cell line, zymography, and serum and urine determinants.

Results: Data showed that oral and/or i.p. administration of M2000 significantly reduces paw edema and histopathological parameters in arthritic rats. The immunosuppressive property of M2000 could significantly diminish clinical signs and histological erosions in the EAE model. Lymph node cell proliferation assay in EAE confirmed the immunosuppressive efficacy of the tested drug. Our findings in ICG and experimental nephrosis showed that M2000 enables a significant decrease in proteinuria, BUN, serum creatinine and cholesterol, as well as glomerular lesion in M2000-treated rats. Moreover, this drug inhibited MMP-2 activity. The pharmacotoxicology study showed that M2000 is the safest anti-inflammatory and immunosuppressive drug in comparison with dexamethasone and conventional NSAIDs tested. Additionally, M2000 had no ulcerogenic effect on the rat stomach.

Conclusions: M2000 is the first novel designed NSAID with the lowest molecular weight, no gastro-nephrotoxicity, and therapeutic effects in glomerulonephritis and nephrosis and could be strongly recommended on an extensive scale as the safest drug for decreasing anti-inflammatory reactions.

key words: **NSAIDs • anti-inflammatory • arthritis • MMP • multiple sclerosis • nephritis • nephrosis**

Full-text PDF: http://www.medscimonit.com/fulltxt.php?IDMAN=6540

Word count: 4670
Tables: 2
Figures: 14
References: 39

Author's address: Prof. Abbas Mirshafiey, Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran-14155, Box: 6446, Iran; e-mail: a_mirshafiey@yahoo.com

BACKGROUND

The pharmacodynamic and pharmacokinetic properties and the therapeutic potential of nonsteroidal anti-inflammatory drugs (NSAIDs) have been well described in the last several decades [1–5]. However, a major concern in recommending NSAIDs for therapeutic purposes is their gastronephrotoxicity [6–10], such that dyspeptic symptoms are estimated to occur in 10–60% of NSAIDs users and lead to discontinuation of treatment in 5–15% of rheumatoid arthritis patients taking NSAIDs [11]. It is now well established that the point prevalence of peptic ulcer disease in patients receiving conventional NSAID therapy ranges between 10 and 30%, representing a 10- to 30-fold increase over that found in the general population [11]. Thus NSAIDs posses not only therapeutic value, but also adverse effects, mainly on the gastrointestinal tract [12]. For this purpose recent attempts have been made to establish a national evidence-based guideline for the prevention of gastric damage induced by nonsteroidal anti-inflammatory drugs. The goal of the guideline is to reduce the number of gastric ulcers with perforation or bleeding as a consequence of NSAID use. It is estimated that 165 patients died as a result of these complications in the year 2000 [13]. Although acetylsalicylic acid (ASA), for example, has been shown useful in various cardiovascular disorders, it can be a major cause of iatrogenic gastrointestinal injury [14]. In addition, NSAIDs, in spite of their therapeutic utility, are known to cause significant renal toxicity, a circumstance that limits their use [7,15,16].

The side effects produced in these organs have been attributed mainly to the inhibitory effect of these drugs on the activity of cyclooxygenase, a key enzyme in prostaglandin synthesis. In addition to this, one of the mechanisms by which NSAIDs induce renal damage is through reactive oxygen species (oxygen free radicals), possibly generated by activated neutrophils and mitochondrial dysfunction [8,15]. Thus, hemodynamic renal failure may result from drugs that reduce renal prostaglandins and hence renal blood flow and glomerular filtration rate [7]. The aim of this investigation was to test the therapeutic potency of M2000 (β-D-mannuronic acid), a novel designed NSAID in experimental models of rheumatoid arthritis, multiple sclerosis, immune complex glomerulonephritis (ICG), and nephrotic syndrome(NS) and its biocompatibility under *in vivo* and *in vitro* conditions.

MATERIAL AND METHODS

Induction of arthritis and treatment protocol

A total of 56 male Lewis rats (125–150 g, Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purpose (D. M. 116192). Rats were divided at random into an i.p. group (n=5×8) with five subgroups (N: normal, C: control, T1: treatment with M2000 by i.p. injection, T2: treatment with indomethacin, H: healthy group receiving M2000) and an oral group (n=2×8) with two subgroups (C: control, T3: treatment with M2000 by oral administration). Adjuvant-induced arthritis (AIA) was developed in Lewis rats by a single intradermal injection (0.1 ml) of heat-killed *Mycobacterium tuberculosis* H37Ra (0.3 mg) in Freund's incomplete adjuvant into the right food pad. Fourteen days after injection of adjuvant, the contralateral left footpad volume was measured with a water displacement plethysmometer. Animals with paw volumes 0.37 ml greater than normal paws were then randomized into the treatment groups. Intraperitoneal administration of the test drugs (M2000, 40 mg/kg/day to group T1 and indomethacin, 2 mg/kg/day to group T2) and oral administration of M2000 (40 mg/kg/day to group T3) were started on day 15 post-adjuvant injection and continued until the final assessment on day 25. During this period, contralateral paw volume measurements were taken intermittently. The left hind limb was removed for histological evaluation.

Clinical assessment of AIA

Rats were evaluated daily for arthritis using a macroscopic scoring system: 0 = no signs of arthritis, 1 = swelling and/or redness of the paw or one digit, 2 = two joints involved, 3 = more than two joints involved, and 4 = severe arthritis of the entire paw and digits (Cuzzocrea et al, 2000).

Histological examination and assessment of arthritic damage

On day 25, the animals were sacrificed while they were under anesthesia and the paws and knees were removed and fixed for histological examination, The following morphological criteria were considered: score = 0, no damage; score = 1, edema; score = 2, inflammatory cell presence; and score = 3, bone resumption.

Pharmacotoxicology study

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 i.p. injections of M2000 (30 mg/kg/48 h) based on the measurements of serum creatinine, BUN, urinary protein excretion, urine urea, as well as the plasma concentrations of triglyceride and cholesterol.
2. Histological examination: renal tissue was 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. Gastro-ulcerogenic study: after i.p. injections of M2000 (30 mg/kg/48 h) to group H, as well as following oral administration of M2000 (40 mg/kg/day) during 10 days to group T3, the animals were killed and the stomach and duodenum 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 i.p. injections of M2000 (30 mg/kg/48 h) was monitored continuously by thermoelements and compared with the baseline temperature in normothermic controls.

Induction of EAE and treatment protocol

A total of 32 six-week-old female Lewis rats were used. Each rat was injected in the hind footpad with 50 µg of MBP in CFA (0.2 ml) containing 500 µg/rat *Mycobacterium tuberculosis*. M2000 was dissolved in saline and administered daily by intraperitoneal (i.p.) injections at a dosage of 40 mg/kg rat body weight. Rats were separated into 2 groups: EAE(M), M2000-treated rats (n=7) received a total of 18 i.p. injections from one day before immunization (day –1) to day 16 post-immunization. EAE(control) rats (n=8) received saline i.p. injections during the same period (from day –1 to day 16).

Clinical evaluation of EAE

All rats were weighed and examined daily for neurological signs as previously described [17] according to the following criteria: grade 0, no disease; grade 1, decreased tail tone or slightly clumsy gait; grade 2, tail atony and/or moderately clumsy gait; grade 3, paraplegia; grade 4, paraplegia with forearm weakness; grade 5, moribund state or death. The data are plotted as the mean clinical score.

Proliferation assay

On day 21 post-immunization, the draining lymph nodes were removed from 4 rats in each group. Lymph node cells were placed in Hanks' solution, and forced through mesh screens to yield a lymph node cell suspension. This cell suspension was tested for proliferative responses to MBP (5 or 10 µg/ml) and mitogen (Con A, 2 µg/ml). Proliferative responses were quantified with the Cell Proliferation ELISA kit (Boehringer Mannheim, Germany).

Neurohistopathological evaluation

Animals were anesthetized with pentobarbital and perfused transcardially with lactated Ringer's solution followed by 4% paraformaldehyde. Paraffin sections of brain and spinal cord were used to assess the degree of inflammation. Histopathological assessment was scored based on the intensity of demyelination, parenchymal damage, necrosis, and perivascular cellular infiltrates in the obtained sections in treated and untreated EAE rats. The neurohistological lesions were quantitated visually by two blinded observers. The histopathological score per CNS segment was defined as the mean numbers of vessels with cellular infiltrates from two examined sections, which had been cut at least in five separate sections when the specimens were processed.

Induction of ICG and treatment protocol

A total of 58 female Sprague Dawley rats weighing 180±20 g were obtained from the Razi institute and were housed in our animal facility (temperature: 20–22°C, humidity: 55–56%, 12-h light/12-h dark cycle, unlimited access to food and water) for at least 1 week before the experiment. Rats were divided randomly into five groups. N: normal group (n=8), P: patient group (n=9), T1 and T2: treated patient groups with M2000 and piroxicam, respectively (n=2×8), and H: healthy controls receiving M2000 (n=8). Immune complex glomerulonephritis was induced by the method of Yamamoto (Yamamoto et al, 1978). Briefly, 8 weeks after subcutaneous immunization with 1 mg of bovine serum albumin (BSA) in complete Freund's adjuvant, the animals received an intravenous dose of 2 mg BSA daily for 4 weeks. On day 56, a therapeutic protocol was developed by i.p administration of M2000 (30 mg/kg) to group T1 and i.p injection of piroxicam (0.3 mg/kg) to group T2. A total of 15 i.p injections at regular 48-h intervals was considered for M2000 and/or piroxicam. The experiment was terminated on day 84 by killing the rats.

Measurement of anti-BSA antibody

Blood samples obtained from the P, T1, and T2 groups in the 6th, 9th, and 12th experimental weeks were considered for measurement of anti-BSA antibody using the ELISA method.

Assessment of kidney function in ICG

Measurement of proteinuria was carried out over six stages, from weeks 3 to 12 post-immunization. Urine was collected before the i.p. injections of M2000 and/or piroxicam. Urine protein was measured using precipitation by trichloroacetic acid. Creatinine clearance was calculated by measuring creatinine concentrations in urine and plasma by the alkaline picrate method. Urine urea and blood urea nitrogen (BUN) was assessed by the oxime method (Evans, 1968).

Histological examinations in ICG

Kidney specimens were processed using light and immunofluorescence microscopic examination. For light microscopy, renal tissues were fixed by immersion in 10% buffered formalin, embedded in paraffin, and 4-µm sections were stained with hematoxilin-eosin and periodic acid-Schiff. The severity and extent of glomerular lesions were blindly evaluated according to four parameters: hypercellularity; glomerular infiltration of PMN; fibrinoid necrosis, and interstitial infiltration. In addition, the existence of tubular casts was considered. These parameters were evaluated by a semi-quantitative method of renal histology using a grading scale of 0–3 (0 = negative, 1 = mild, 2 = moderate, 3 = severe).

Induction of NS and treatment protocol

A total of 41 female Sprague Dawley rats weighing 180±20 g were obtained from Razi institute and housed in our animal facility for at least one week before the experiment. The rats were divided at random into five groups, N: normal group (n=8), P: patient group (n=9), T1 and T2: treated patient groups with M2000 and piroxicam, respectively (n=2×8), and H: healthy controls receiving M2000 (n=8). To induce nephrotic syndrome, adriamycin (Adriablastina; Farmitalia, Milan, Italy) was given once by a single intravenous injection (7.5 mg/kg) through the tail vein. Six days after injection of adriamycin, a therapeutic protocol was developed by i.p administration of M2000 (30 mg/kg) to group T1 and i.p. injection of piroxicam (0.3 mg/kg) to group T2. Total i.p. injections were 14, of which five were made every day and nine were carried out at regular 48-h intervals. The therapeutic protocol was terminated on day 28 and the animals were killed on day 43. The normal group

(N) received nothing and group (H) was healthy controls receiving M-2000.

Assessment of kidney function in NS

Measurement of proteinuria was done over seven stages. At the start of treatment, urine was collected before the i.p. injections of M2000 and/or piroxicam. Urine protein was measured using precipitation by trichloroacetic acid. Creatinine clearance was calculated by measuring creatinine concentrations in urine and plasma by the alkaline picrate method. Urine and blood urea nitrogen (BUN) were assessed by the Veniamin method [18].

Evaluation of serum lipid levels

Serum triglyceride and cholesterol were determined by routine laboratory tests on the day of sacrifice.

Measurement of cytokine

Assessment of the serum level of interleukin-6 (IL-6) was carried out using the enzyme immunoassay kit ER2-IL-6 (Lot No. DA 53152 CA, Endogen Co, USA).

Renal histopathological studies in NS

Kidney specimens were processed using light microscopy. Renal tissues were fixed by immersion in 10% buffered formalin, embedded in paraffin, and 4-micron sections were stained with hematoxylin-eosin and periodic acid-Schiff. The severity and extent of glomerular lesions were blindly evaluated according to four parameters: hypercellularity, glomerular infiltration of PMN, tubular casts, and cellular swelling (hydropic change) in the capillary network within the renal cortex. These parameters were evaluated by a semi-quantitative method of renal histology using a grading scale of 0–3 (0 = negative, 1 = mild, 2 = moderate, 3 = severe).

Cell culture

The fibrosarcoma WEHI-164 cell line was seeded at an initial density of 20,000 cells/well in a 96-well tissue culture plate. The cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 µg/ml, with 5% CO_2, at 37°C, and in saturated humidity.

Dose-response analysis

Triplicate, two-fold dilutions of M2000, dexamethasone, piroxicam, and diclofenac preparations at concentrations of 10–200 µg/ml were transferred to overnight cultured cells. Untreated cells were used as controls. The cells were cultured overnight and then subjected to colorimetric assay. A sample of the media was used for zymography.

Colorimetric assay

After each experiment, the cells were washed three times with ice-cold PBS followed by fixation in a 5% formaldehyde solution. The fixed cells were washed three times and stained with 1% crystal-violet. The stained cells were washed, lysed, and solublized with a 33.3% acetic acid solution. The density of the developed purple color was read at 580 nm.

Zymoanalysis

This technique was used for determining matrix metalloproteinase type 2 (MMP-2) and MMP-9 activity in conditioned media according to the modified Heussen and Dowdle method [19]. Using a UVI Pro gel documentation system (GDS_ 8000 System), quantitative evaluation of both the surface and intensity of lysis bands, on the basis of grey levels, were compared relative to untreated control wells and expressed as the "relative expression" of gelatinolytic activity.

Statistical analysis

All data are expressed as the mean ± SD. The significance of difference in arthritic and EAE score as well as histopathology data were evaluated with the Mann-Whitney test. The parametric data were compared using the student's *t*-test. Differences were considered significant at $P<0.05$.

RESULTS

Effect of i.p. administration of M2000 in AIA

Figure 1 shows a time-dependent increase in left hind paw volume (ml) in rats induced by adjuvant. The incidence rate of arthritis in animals was 100% (8/8). Interaperitoneal injection of M2000 to arthritic rats could rapidly reverse paw edema, as did indomethacin. The difference between the control group and treated rats on days 20–25 of the experiment was significant ($P<0.05$).

Effect of oral administration of M2000 in AIA

In Figure 2, oral administration of M2000 (40 mg/kg) 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 ($P<0.05$). There was no macroscopic evidence of either hind paw erythema and/or edema in the normal control rats.

Histopathology findings in AIA

As shown in Figure 3A,B, histological evaluation of the paws in the vehicle-treated arthritic animals revealed signs of severe arthritis along with inflammatory cells infiltrate. Histopathological assessment showed reduced inflammatory cell infiltrate in the joints of treated rats as well as a reduced number of osteoclasts present in the subchondral bone; tissue edema and bone erosion in the paws were also markedly reduced by both treatments (M2000 and/or indomethacin), indicating that the tested drugs were effective in retarding synovial inflammation and prevented destruction of joints.

Pharmacotoxicological findings

Data analysis showed no significant differences in the levels of serum determinants (BUN, creatinine, cholesterol, and triglyceride) and urine determinants (protein excretion and

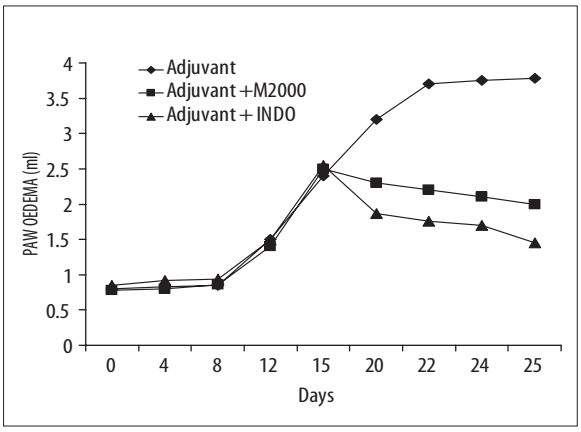

Figure 1. Severity of arthritis score in treated groups (A+M) compared with untreated control rats (A). This figure demonstrates a time-dependent increase in left hind paw volume (ml) in rats induced by adjuvant. The interaperitoneal injection of M2000 to arthritic rats could rapidly reverse paw edema, as did indomethacin. The difference in paw swelling between treated and untreated rats on days 20–25 was significant ($P<0.05$), whereas there was no significant difference in decreasing the severity of arthritis between M2000 and indomethacin-treated animals.

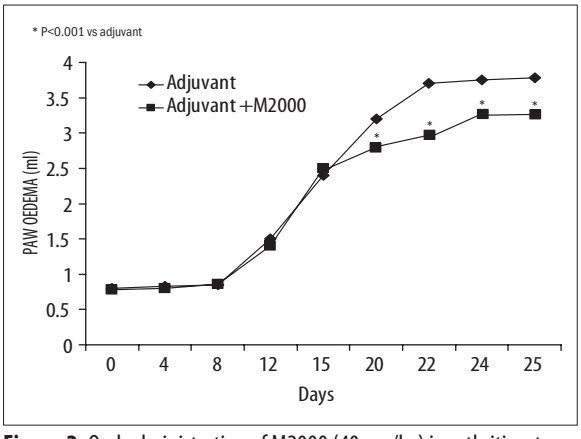

Figure 2. Oral administration of M2000 (40 mg/kg) in arthritic rats induced a significant ($P<0.05$) reduction in paw edema. After 10 days of treatment, paw swelling was reduced in M2000-treated animals relative to the paw volume of vehicle-treated rats. This difference was statistically significant ($P<0.05$).

creatinine) between the normal group (N) and healthy experimental group (H) challenged with M2000 (Table 1), whereas the significant increase of urine urea in group H compared with normal group (N) revealed the advantage of the use of M2000 in healthy controls (Table 1). Histologic study of kidney specimens obtained from the normal group and healthy controls receiving M2000 showed no glomerular changes in group H 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 test drug. Additionally, the results of this experiment showed that 12 i.p. injections of M2000 (30 mg/kg/48 h) as well as oral administration of

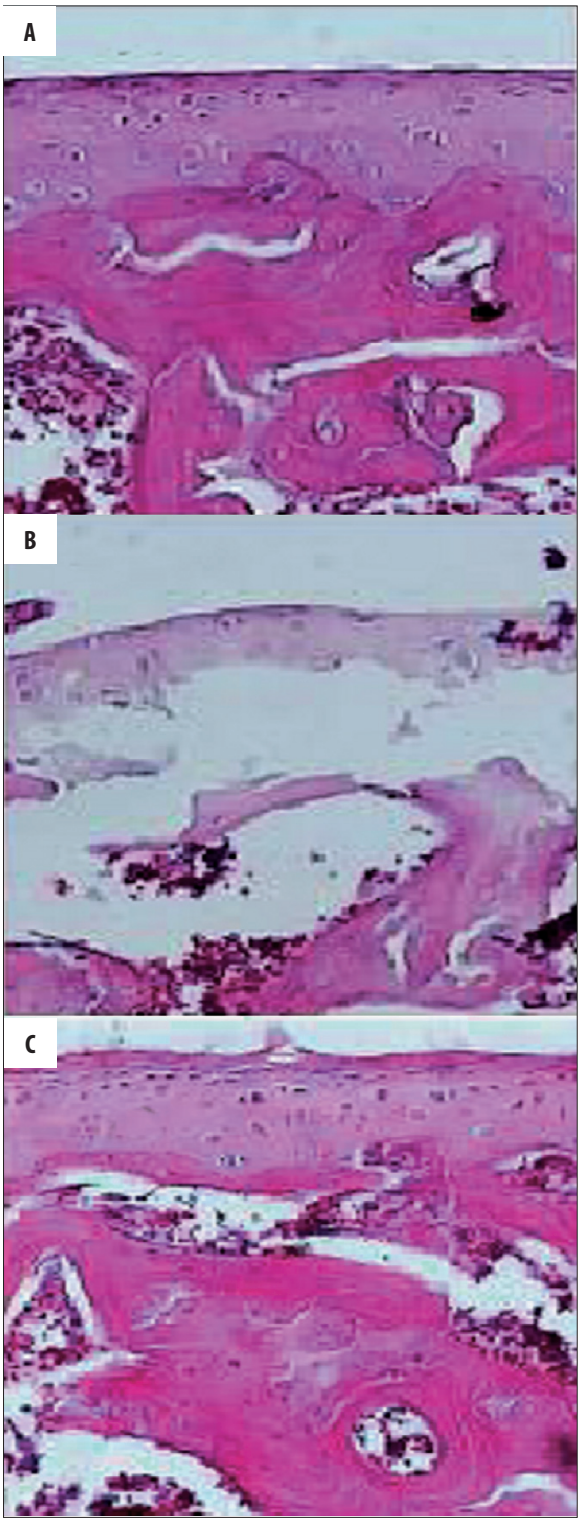

Figure 3A. Representative histopathological slides of a hind limb joint of a healthy Lewis rat (**A**), an animal with adjuvant-induced arthritis (**B**), and an arthritic animal treated with M2000 (**C**). The joint of the rat which was treated with M2000 (**C**) shows significantly fewer signs of joint destruction.

M2000 (40 mg/kg/day) over 10 days could not provoke gastromucosal lesions in the rats of experimental group.

Table 1. Pharmacotoxicological effect of M2000 on serum (S) and urine (U) determinants.

Group	No Rat	BUN mg/dl	S.Creat mg/dl	S.Chol mg/dl	S.Trig mg/dl	U.Pro mg/24 h	U.Urea mg/dl
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	1284±226

Healthy group receiving M2000, 30 mg/kg (H), versus normal (N) group showed no significant differences in serum and urine determinants.

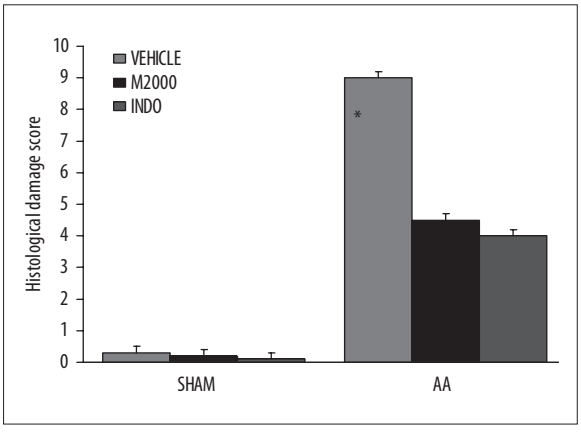

Figure 3B. Effect of M2000 treatment on histological damage score. The histological evaluation of the paws in the vehicle-treated arthritic animals reveals signs of severe arthritis along with inflammatory cell infiltrate. M2000 therapy could significantly reduce this pathological parameter compared with the control group. $P<0.05$ was considered significant.

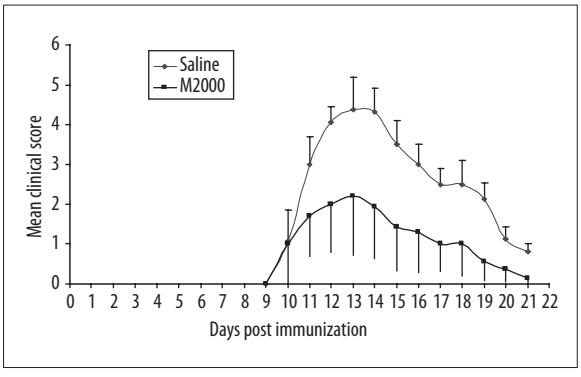

Figure 4. Amelioration of MBP-induced EAE by preventive application of M2000. Fifteen Lewis rats were immunized with 50 µg MBP in CFA into one hind footpad and then divided into two groups. M2000-treated rats (n=7) received a total of 18 i.p. injections M2000 (40 mg/kg/day) from one day before immunization to day 16 post-immunization. Control rats (n=8) received saline i.p. injections during the same period. M2000 therapy caused a significant reduction in clinical score compared with untreated control ($P<0.05$). Bars indicate SD.

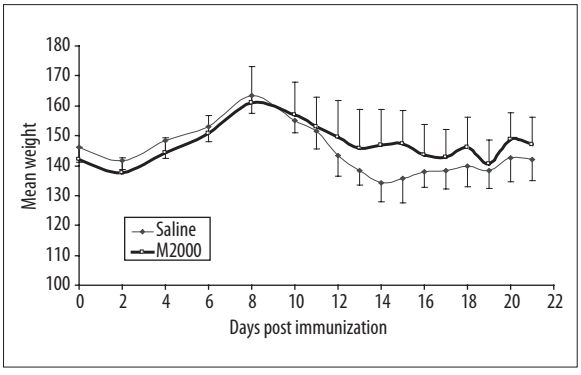

Figure 5. This figure shows the higher weight gain in M2000-treated rats compared with untreated controls during the EAE period. M2000-treated rats (n=7) received a total of 18 i.p. injections M2000 (40 mg/kg/day) from one day before immunization to day 16 post-immunization. Control rats (n=8) received saline i.p. injections during the same period. Bars indicate ±SD.

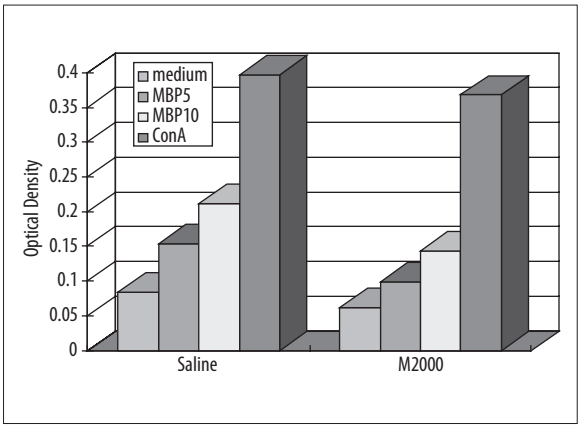

Figure 6. The graph shows the effect of M2000 at two different doses (5 and 10 µg/ml) on the proliferative responses of regional lymph node cells from EAE rats (n=4 for each group, on day 21 after immunization).

Immunosuppressive effect of M2000 in EAE

Following the chronological changes in clinical score of each group of the EAE rats (Figures 4, 5), the first clinical signs appeared in some rats of each group on day 10, and then symptoms reached their maximum on day 13 post-immunization. Maximum severity of EAE was a score of 5 in each group. The rats treated with M2000 were less affected than the controls (Figure 4). Three of EAE(M) rats did not develop apparent signs of EAE during the period of observation, whereas all of EAE(control) rats developed typical signs (clinical score >4) of EAE. The mean clinical scores of EAE(M) rats were significantly milder than those of EAE(control) rats on days 11 to 19 (two-way repeated measures of ANNOVA, $P<0.05$). Mean body weight was more reduced in EAE(control) rats than in EAE(M) rats after day 12 (Figure 5).

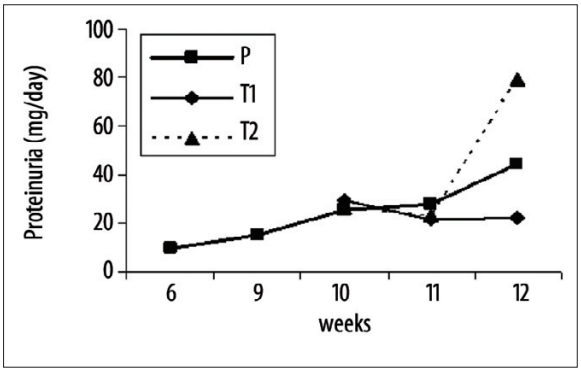

Figure 7. Time course of the mean values of proteinuria in the groups N, P, T1, and T2 in immune complex glomerulonephritis. N: Normal rats (n=8), P: Patient rats (n=9), T1 and T2: Treated groups with intraperitoneal injections of M2000 (30 mg/kg) and piroxicam (0.3 mg/kg), respectively (n=2×8). Onset of i.p administration of M2000 and piroxicam to the T1 and T2 groups was day 56. The number of i.p injections was 15, injection interval 48 h, and the end of the therapeutic protocol was day 84. There was significant difference between the M2000-treated group (T1) and the patient group. $P<0.05$ was considered significant.

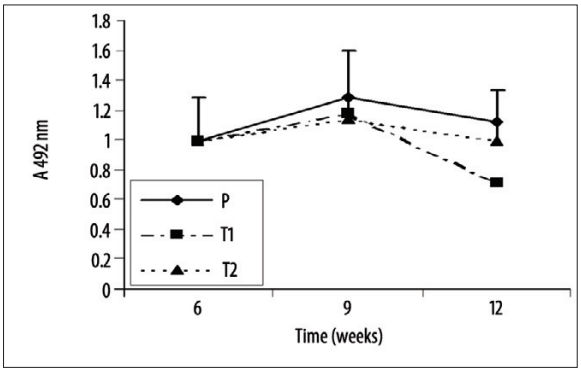

Figure 8. Anti-BSA antibody titers in groups P, T1, and T2 during the course of acute BSA-nephritis as determined by an ELISA method. The highest titer occurs in P, the intermediate in T2, and the lowest in T1. Values are mean ±S.D. The difference between the M2000-treated group (T1) and the patient group (P) in the 12th week was significant. $P<0.05$ was considered significant.

PI

The MBP-specific proliferative response of draining lymph node cells

Lymph node cells from the rats treated with M2000 exhibited a reduced proliferation to MBP in contrast to those of EAE(control) rats, whereas the proliferative responses of lymph node cells to Con A were almost the same in both groups (Figure 6).

Neuropathologic Evaluation in EAE

Histopathological assessment of stained sections of brain and spinal cord exhibited a little demyelination, parenchymal damage, and necrosis in EAE(control) (untreated) rats. Therefore, semi-quantitative assessment of histology, transverse brain and longitudinal spinal cord sections, was established to determine the presence of mononuclear cell infiltrates in the perivascular spaces. Neurohistopathological evaluation of brain and spinal cord sections in the treated and untreated 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 significantly less than in untreated controls, which had mean values of (2.43±3.41) for brain and (10.71±8.76) for spinal cord.

Effect of M2000 on serum and urine determinants in ICG

The changes in the mean levels of urinary protein excretion between the N, P, T1, and T2 groups are shown in Figure 7. Urinary protein excretion was significantly less in M2000-treated animals than in untreated controls ($P<0.05$). Here, reduction in proteinuria in T1 vs. the P and T2 groups was significant ($P<0.05$). BUN, serum cholesterol, and triglyceride levels were elevated in the P and T2 groups compared with M2000-treated rats, but these differences were not significant. Also, there were no significant differences between T1 vs. the P and T2 groups in urine urea and creatinine, whereas the difference between T1 vs. the P and T2 groups in reduction of proteinuria was significant ($P<0.05$). Here, decreased serum determinant concentrations and increased urine determinant (urea and creatinine) levels following M2000 therapy paralleled the amelioration process of the disease.

Anti-BSA antibody titer in ICG

Changes in the mean levels of anti-BSA antibody titers in the 6th, 9th, and 12th experimental weeks are illustrated in Figure 8. Anti-BSA antibody titers were significantly lower in M2000-treated animals (T1) than in piroxicam (T2) and untreated controls (P) at the end of the experiment (day 84).

Histopathological findings in ICG

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

Effect of M-2000 on renal function in NS

The changes in the mean levels of urinary protein excretion between N, P, T1, and T2 are shown in Figure 9. This experiment showed that i.p. administration of M2000 (30 mg/kg) could exert its therapeutic effects on adriamycin-induced nephropathy. For an exact evaluation of M2000's effects, the experiment was terminated 14 days after the last M2000 injection. Urinary protein excretion was significantly less in M2000-treated animals compared with untreated controls and piroxicam-treated animals ($P<0.05$). Here, T1 vs. P was significant. In Figure 10 (A) the amounts of BUN are compared between the different groups (N, P, T1, and T2).This figure shows that the difference between treated

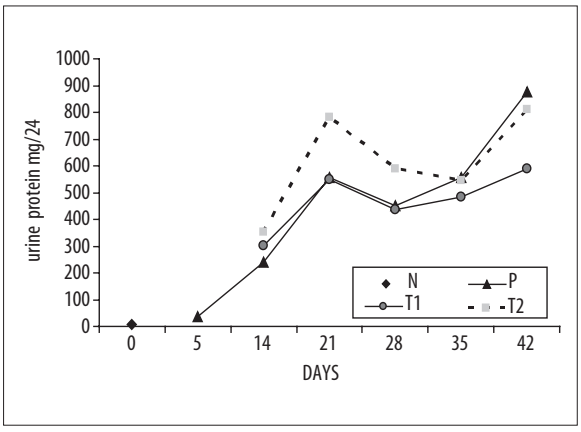

Figure 9. Time course of the mean values of proteinuria in different groups (N, P, T1, T2) in experimental nephrosis. N: normal rats (n=8), P: patient (non-treated) rats that show an increase of proteinuria levels after day 5 (n=9), T1, T2: treated groups by i.p. injections of M2000 (30 mg/kg) and piroxicam (0.3 mg/kg), respectively (n=2×8). Note: a single i.v injection of adriamycin (7.5 mg/kg body weight) induced a severe nephrotic syndrome. Onset of i.p. administration of M2000 and piroxicam in the T1, T2 groups was day 6 (after development of disease). There were significant differences between the untreated (P) and M2000-treated rats (T1) groups. $P<0.05$ was considered significant.

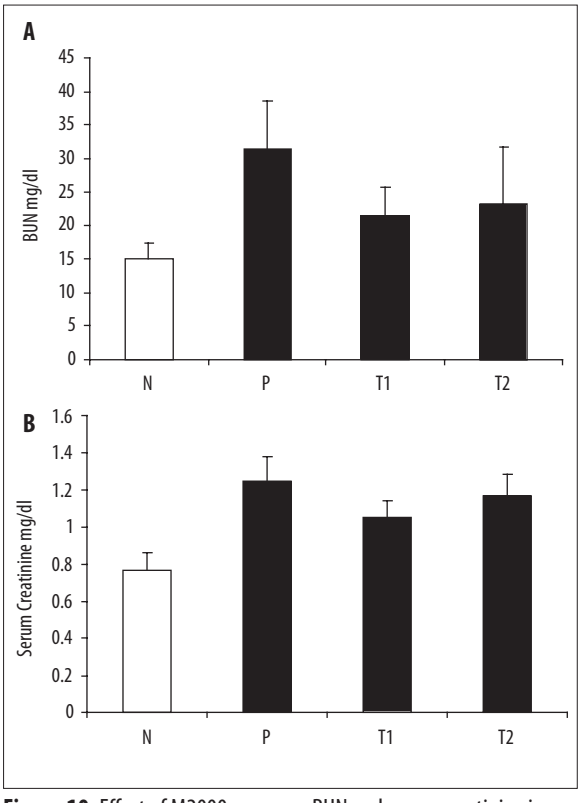

Figure 10. Effect of M2000 on serum BUN and serum creatinine in groups N: normal (8), P: patients (9), T1: treated with M2000 (8), T2: treated with piroxicam (8). Each bar represents the mean ±SD. (**A**): BUN concentration in groups N, P, T1, and T2. T1 vs. P was significant. ($P<0.05$). (**B**): Creatinine concentration in groups N, P, T1, and T2. T1 vs. P was significant. ($P<0.05$).

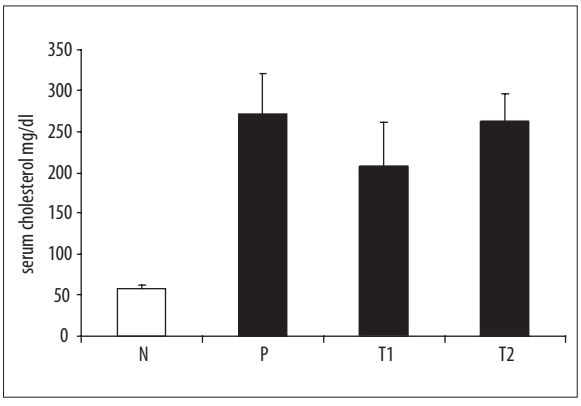

Figure 11. Effect of M2000 on serum cholesterol level in groups N: normal (8), P: patients (9), T1: treated with M2000 (8), T2: treated with piroxicam (8). Each bar represents the mean ±SD. Cholesterol concentration in group T1 vs. P was significant. ($P<0.05$).

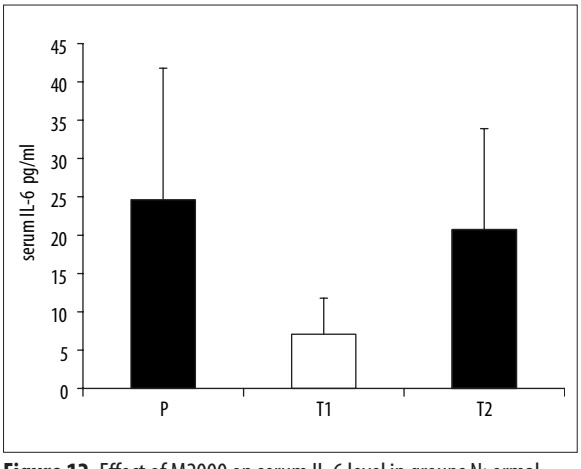

Figure 12. Effect of M2000 on serum IL-6 level in groups N: ormal (8), P: patients (9), T1: treated with M2000 (8), T2: treated with piroxicam (8). Each bar represents the mean ±SD. Difference between groups T1 vs. P in IL-6 concentration was significant. ($P<0.05$).

rats (T1) and untreated animals (P) is significant ($P<0.05$). In Figure 10 (B) the comparison of serum creatinine concentration among various groups shows that there is significant difference between T1 vs. P ($P<0.05$), whereas there were no significant differences in the levels of urinary urea nitrogen and urine creatinine concentration between M2000-treated animals and untreated controls (data not shown).

Effect of M2000 on serum lipid levels in NS

Serum cholesterol and triglyceride levels were significantly elevated (P at least <0.05) in nephrotic rats compared with the healthy controls at the end of experiment. Intraperitoneal injections of M2000 to patient rats significantly reduced serum cholesterol in nephrotic animals (Figure 11).

Effect of M-2000 on IL-6 production

Figure 12. Shows the effect of M2000 therapy in reducing IL-6 production in treated patient rats (group T1). The dif-

Table 2. Light-microscopic findings of kidney histological lesions in various groups.

Group	n	Hypercellularity	PMN Infiltration	Cast	Hydropic change
N	8	0.40±0.53	–	–	–
P	9	1±0.57	1.71±0.48	2.14±0.69	1±0
T1	8	0.57±0.53	1	1.14±0.63	–
T2	8	0.71±0.46	1.6±0.76	1.87±1.1	–
H	8	0.42±0.53	–	–	–

Values are expressed as Mean ±SD. Semiquantitative scoring of histological lesion show significant differences between groups P and T1 in hypercellularity, glomerular PMN infiltration, hydropic change in the capillary network within the renal cortex, and in tubular casts ($P<0.05$).
Note: Group H was the healthy control receiving M2000 (30 mg/kg).

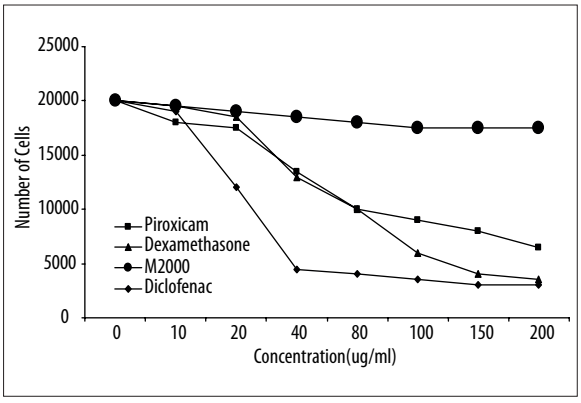

Figure 13. Cytotoxic effect of M2000. Proliferative response of a fibrosarcoma (WEHI-164) cell line to M2000 at different doses (10–200 µg/ml) compared with diclofenac, piroxicam, and dexamethasone. LD_{50} for diclofenac, piroxicam and dexamethasone was 25, 80, and 80 µg/ml, respectively. In contrast, WEHI-164 as a sensitive cell line showed a high tolerability against increasing amounts of M2000.

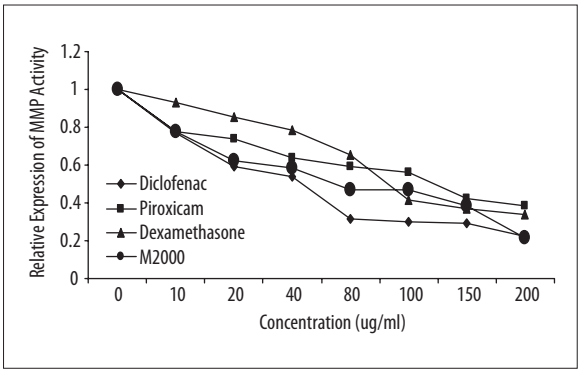

Figure 14. Inhibitory effect of M2000 on MMP-2 activity. Fibrosarcoma cell lines (2×10^4 cells/well) were incubated overnight with increasing dose of M2000 as described in Materials and Methods. Diclofenac-, piroxicam-, and dexamethasone-treated cells were used as controls. M2000 treated and untreated cells were investigated in triplicate. The inhibitory activity of M2000 paralleled diclofenac's at doses of 10, 20, 40, and 200 µg/ml, whereas the inhibitory effect of M2000 at concentrations of 20, 40, and 80 µg/ml was significantly more than dexamethasone's ($P<0.05$). Moreover, the inhibitory activity of this novel agent at a dose of 200 µg/ml was significantly more than those of dexamethasone and piroxicam ($P<0.05$).

ference between the groups of T1 and P in IL-6 concentration was significant, ($P<0.05$).

Histopathological findings in NS

Light microscopic 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 animals treated with M2000 showed a significant reduction in glomerular changes compared with untreated controls (Table 2).

Biocompatibility of M2000

Figure 13 shows the proliferative response of the fibrosarcoma WEHI-164 cell line to M2000 at different doses (10–200 µg/ml) compared with 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, respectively. M2000 showed no cytotoxic effect compared with steroidal and nonsteroidal tested drugs.

Effect of M2000 on MMP-2 activity

Dose response analysis of M2000 on MMP-2 activity is presented in Figure 14. The inhibitory effect of M2000 at concentrations of 20, 40, 80, and 200 µg/ml was significantly more than that of dexamethasone ($P<0.05$) and this difference was significant between M2000 and piroxicam at the concentration of 200 µg/ml, ($P<0.05$), whereas the inhibitory activity of this novel agent paralleled diclofenac's at doses 10, 20, 40, and 200 µg/ml.

Discussion

This investigation was planned to explore the anti-inflammatory and immunosuppressive properties and gastro-nephrotol-

erability of M2000 (β-D-mannuronic acid), a novel designed NSAID, in various experimental models. Nonsteroidal anti-inflammatory drugs have been widely used for the modulation of the inflammatory response. However, a number of facts involve the occurrence of gastrointestinal and renal damage caused by NSAIDs [7,20,21]. Among this group of drugs, acetylsalicylic acid has been fully studied [22–24]. The mechanism of action of NSAIDs seems to be multifactorial and is not limited to inhibition of cyclooxigenases [25]. Other mechanisms which are possibly involved in the anti-inflammatory action of NSAIDs include inhibition of neutrophil aggregation and superoxide production, induction of the shedding of L-selectin, decrease in proteoglycanase activity, and gelatinase release [1,4,5,26]. MMPs, especially the gelatinases (MMP-2 and MMP-9), are implicated in several features of inflammatory arthritis, including angiogenesis and bone erosions [27], because angiogenesis is a major component of the inflammatory pannus in rheumatoid arthritis. MMP secretion by microvascular endothelial cells is an essential step in angiogenesis [28]. The role of M2000 in the reduction of MMP-2 activity could be similar to the efficacy of TGF-β1, sulfasalazine, and its metabolites in lowering the levels of MMP-2 activity in inflamed joints [29,30]. On the other hand, mesangial cells play a prominent role in renal inflammatory disorder, matrix metalloproteinases (MMPs) play a role in the activation of mesangial cells, and increased cell proliferation rates and extracellular matrix accumulation are crucial targets in the therapy of glomerulonephritis [31–33]. Therefore, MMP inhibitors provide a new approach to the therapy of inflammation, probably even beyond the field of renal disorders [33–35]. Our findings using the zymoanalysis method showed that M2000 is a potent MMP inhibitor. In contrast, the result of treatment of glomerulonephritis with piroxicam paralleled the administration outcomes of other NSAIDs [7,15,36], in which the use of piroxicam could reinforce renal insufficiency in our experimental model, whereas M2000 not only did not exacerbate renal damage, but, interestingly, this novel designed NSAID could significantly diminish renal lesions in the experimental model of glomerulonephritis.

The results of M2000 therapy in experimental rheumatoid arthritis and multiple sclerosis revealed the ant-inflammatory and immunosuppressive properties of this novel NSAID. The most important advantage of M2000 compared with conventional NSAIDs is its having the lowest molecular weight, no gastro-nephrotoxicity, and its being a safe and natural molecule. In addition, it has been reported that M2000 has an anti-tumoral property due to its apoptotic efficacy [37]. Based on the molecular structure of M2000, it could be strongly predicted that the cell surface receptors for M2000 may be the mannose receptor (MR), Endo 180/uPARAP from the mannose receptor family, and probable accessory receptors for this molecule will be Toll-like receptor 2 (TLR2), TLR4, CD11b/CD18 (Mac-1 or CR3), and P-selectin. On the other hand, the involvement of macrophage MR in the binding and transmission of HIV by macrophages [38] as well as the fact that tissue loss during aging and age-dependent pathogenesis are the result of a disturbed regulation of proteolytic activity, MMP-2 and -9 activities especially should also be considered [39]. Thus we predict that M2000, in addition to its anti-inflammatory, immunosuppressory and anti-tumoral properties, may, following further investigation, be recommended as an anti-aging drug, nutritional supplement, and provide a possible survival benefit in HIV positive individuals.

CONCLUSIONS

Our data suggest that M2000 (β-D-mannuronic acid), as a new anti-inflammatory drug with immunosuppressive property and the greatest tolerability, not only shows no gastro-nephrotoxicity, but it is the first novel designed NSAID with the lowest molecular weight and with therapeutic effects in glomerulonephritis and nephrosis and could be strongly recommended on an extensive scale as a the safest drug for decreasing anti-inflammatory reactions.

Acknowledgements

We wish to thank the Minister of Science and Research of North-Rhine Westphalia, Germany, Dr. Hannelore Kraf for her award to M2000. The authors would like to thank Dr. Farshid Saadat for his excellent technical assistance.

REFERENCES:

1. Abramson S, Weissmann G: The mechanism of action of nonsteroidal anti-inflammatory drugs, Clin Exp Rheumatol, 1989; 7: 163–70
2. McCormack K, Brune K: Dissociation between the antinociceptive and anti-inflammatory effects of the nonsteroidal anti-inflammatory drugs: a survey of their analgesic efficacy. Drugs, 1991; 41: 533–47
3. Rhoda Lee C, Balfour JA: Piroxicam-β-Cyclodextrin, A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in rheumatic diseases and pain states. Drugs, 1994; 48: 907–29
4. Gomez-Gaviro MV, Gonzalez-Alvaro I, Dominguez-Jimenez C et al: Structure-function relationship and role of tumor necrosis factor-alpha-converting enzyme in the down-regulation of L-selectin by nonsteroidal anti-inflammatory drugs, J Biol Chem, 2002; 277: 38212–21
5. Vignon E, Mathieu P, Louisot P et al: *In vitro* effect of nonsteroidal anti-inflammatory drugs on proteoglycanase and collagenase activity in human osteoarthritic cartilage, Arthritis Rheum, 1991; 34: 1332–35
6. Fogo AB: Acute interstitial nephritis and minimal change disease lesion, caused by NSAIDs injury, Am J Kidney Dis, 2003; 42: A41–E1
7. Perazella MA: Drug-induced renal failure: update on new medications and unique mechanisms of nephrotoxicity, Am J Med Sci, 2003; 325: 349–62
8. Stolbergen C, Finsterer J: Side effect of conventional nonsteroidal anti-inflammatory drugs and celecoxib: more similarities than differences. South Med J, 2004; 97: 209
9. Laporte JR, Ibanez L, Vidal X et al: Upper gastrointestinal bleeding associated with the use of NSAIDs: newer versus older agents, Drug Saf, 2004; 27: 411–20
10. Dupas JL, Grigy C: Curative and preventive treatment of NSAIDs-associated gastrointestinal ulcers, Gastroentrol Clin Biol, 2004; 28: C77–C83
11. Lazzaroni M, Bianchiporro G: Gastrointestinal side-effects of traditional non-steroidal anti-inflammatory drugs and new formulations, Aliment Pharmacol Ther, 2004; 20: 48–58
12. Bauerova K, Nosalova V, Mihalova D et al: Contribution to safe anti-inflammatory therapy with indomethacin, Cent Eur J Public Health, 2004; 12: S8–S10
13. Moens HJ, van Croonenborg JJ, Al MJ et al: Guideline 'NSAID use and the prevention of gastric damage', Ned Tijdschr Geneeskd, 2004; 148: 604–08
14. Marzo A, Crestani S, Fumagalli I et al: Endoscopic evaluation of the effects of indobufen and aspirin in healthy volunteers, Am J Ther, 2004; 11: 98–102
15. Basivireddy J, Jacob M, Pulimood AB et al: Indomethacin-induced renal damage: role of oxygen free radicals, Biochem. Pharmacol, 2004; 67: 587–99
16. Reinhold SW, Fischereder M, Riegger GA et al: Acute renal failure after administration of a single dose of a highly selective COX-2 inhibitor, Clin Nephrol, 2003; 60: 295–96
17. Matsuo H, Ichinose K, Ohtsuru I et al: Treatment of experimental allergic encephalomyelitis by selective removal of CD4 T cells, Theraputic apheresis, 1997; 1: 165–68

18. Veniamin MP, Vakirtzi-Lemonias C: Chemical basis of the carbamidodiacetyl micromethod for estimation of urea, citrulline and carbamyl derivatives, Clin Chem, 1970; 16: 3–6
19. Heussen C, Dowdle EB: Electrophoretic analysis of plasminogen activator in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates, Anal Biochem, 1980; 102: 196–202
20. Maciel HP, Cardoso LG, Ferreira LR et al: Anti-inflammatory and ulcerogenic effects of indomethacin and tenoxicam in combination with cimetidine. Inflammopharmacology, 2004; 12: 203–10
21. Lamarque D, Chaussade S: Risk factors for upper digestive tract toxicity with NSAIDs (excluding *Helicobacter pylori*). Presse Med, 2003; 32: S56–S59
22. Darling RL, Romero JJ, Dial EJ et al: The effects of aspirin on gastric mucosal integrity, surface hydrophobicity, and prostaglandin metabolism in cyclooxygenase knockout mice. Gastroenterology, 2004; 27: 341–43
23. Yetkin G, Celebi N, Ozer C et al: The healing effect of TGF-alpha on gastric ulcer induced by acetylsalicylic acid in rats. Int J Pharm, 2004; 277: 163–72
24. Sibilia J, Ravaud P, Marck G: Digestive and hemorrhage complications of low-dose aspirin, Presse Med, 2003; 32: S17–S28
25. Sanchez C, Mateus MM, Defresne MP et al: Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1 beta and nonsteroidal anti-inflammatory drugs. J Rheumatol, 2002; 29: 772–82
26. Sopata I, Wojtecka-Luksik E, Maslinski S: Auranofin modulates gelatinase release from rat neutrophils. Int J Tissue React, 1987; 9: 471–76
27. Hitchon CA, Danning CL, Illei GG et al: Gelatinase expression and activity in the synovium and skin of patients with erosive psoriatic arthritis. J Rheumatol, 2002; 29: 107–17
28. Jackson CJ, Arkell J, Nguyen M: Rheumatoid synovial endothelial cells secrete decreased levels of tissue inhibitor of MMP (TIMP1). Ann Rheum Dis, 1998; 57: 158–61
29. Chernajovsky Y, Adams G, Triantaphyllopoulos K et al: Pathogenic lymphoid cells engineered to express TGF Beta 1 ameliorate disease in a collagen-induced arthritis model. Gene Ther, 1997; 4: 553–59
30. Minghetti PP, Blackburn WD Jr: Effects of sulfasalazine and its metabolites on steady state messenger RNA concentration for inflammatory cytokines, matrix metalloproteinases, and tissue inhibitors of metalloproteinase in rheumatoid synovial fibroblasts. J Rheumatol, 2002; 27: 653–60
31. Lenz O, Elliot SJ, Stetler-Stevenson WG: Matrix metalloproteinases in renal development and disease. J Am Soc Nephrol, 2000; 11: 574–81
32. Chadban S: Glomerulonephritis recurrence in the renal graft. J Am Soc Nephrol, 2001; 12: 394–402
33. Marti HP: The role of matrix metalloproteinases in the activation of mesangial cells. Transpl Immunol, 2002; 9: 97–100
34. Lovett DH, Johnson RJ, Marti HP et al: Structural characterization of the mesangial cell type IV collagenase and enhanced expression in a model of immune complex-mediated glomerulonephritis. Am J Pathol, 1992; 141: 85–98
35. Harendza S, Schneider A, Helmchen U et al: Extracellular matrix deposition and cell proliferation in a model of chronic glomerulonephritis in the rat. Nephrol Dial Transplant, 1999; 12: 2873–79
36. Kaiser A: Diclofenac caused renal insufficiency. A case illustrating the necessity of pharmaceutical intervention and care. Med Monatsschr Pharm, 2003; 26: 384–88
37. Mirshafiey A, Khorramizadeh MR, Saadat F, Rehm BHA: Chemopreventive effect of M2000, a new anti-inflammatory agent. Med Sci Monit, 2004; 10(10): PI105–PI109
38. Nguyen DG, Hildreth JE: Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. Eur J Immunol, 2003; 33: 483–93
39. Isnard N, Peterszegi G, Robert AM et al: Regulation of elastase-type endopeptidase activity, MMP-2 and MMP-9 expression and activation in human dermal fibroblasts by fucose and a fucose-rich polysaccharide. Biomed Pharmacother, 2002; 56: 258–64