Is Taurolidine a candidate for treatment of rheumatoid arthritis?

J. Marcinkiewicz[1], P. Głuszko[2], E. Kontny[4], B. Kwaśny-Krochin[2], M. Bobek[1], W. Wierzchowski[3], M. Ciszek[1], W. Maśliński[4]

[1]Chair of Immunology, [2]Department of Rheumatology, and [3]Chair of Pathomorphology, Jagiellonian University Medical College, Cracow; [4]Department of Immunology, Institute of Rheumatology, Warsaw.

Abstract

Objective

To study the therapeutic potential of taurolidine (TRD), a derivative of taurine with known anti-inflammatory and anti-proliferative properties, in various experimental models of synovitis.

Methods

In vitro*: fibroblast-like synoviocytes (RA FLS) isolated from the synovial tissue of patients with rheumatoid arthritis (RA) were cultured in the presence of either TRD or polyvinylpyrrolidine (PVP), the pharmaceutical stabilizer of TRD, which was used as a control. Proliferation of RA FLS and cytokine (IL-6 and IL-8) release were measured.*

In vivo*: (A). The effect of systemic TRD treatment on the development of collagen-induced arthritis (CIA) in female DBA1/J mice was investigated. Mice were treated either with intraperitoneal injections of 1 ml of 2% Taurolin Boehringer Ingelheim (TRD +PVP) or with PVP as placebo. The incidence of arthritis, myeloperoxidase (MPO) activity in periarticular tissue, as well as serum concentration of IgG specific to collagen II (IgG αCII) were determined.*

(B). The effect of intra-articular TRD treatment was studied in rabbits with antigen-induced monoarthritis (AIA). After the induction of AIA of right knees rabbits were treated either with intra-articular injections of 0.5 ml of 2% Taurolin or 0.5ml PVP (placebo). The animals were examined for clinical signs of arthritis and diameter of joints was measured. After termination of the experiment, the arthritic knees were examined and histopathology of the joints was assessed. In addition, serum amyloid A (SAA) concentration was measured.

Results

In vitro*: TRD exerted cytotoxic effect on RA FLS when applied at concentrations >100 μM. TRD at non-cytotoxic concentrations, inhibited PDGF-triggered RA FLS proliferation, reduced IL-1β – stimulated production of IL-6 and slightly decreased intracellular content of IL-8.* In vivo*: (A). Intraperitoneal treatment with Taurolin significantly reduced the incidence (30%) of CIA when compared to the control mice (79%). However, Taurolin failed to control the development of CIA in mice with high serum level of IgG αCII (>1000 U).*

(B). Intra-articular application of 2% Taurolin resulted in amelioration of AIA in all treated rabbits (reduced diameter of arthritic joints and smaller rise of SAA level as compared to the control animals). Histopathologic evaluation revealed pannus formation in both groups and extensive necrotic lesions of synovial tissue treated with TRD, suggesting synoviorthesis-like effect.

Conclusion

Results from AIA and from in vitro *RA FLS studies suggest that intra-articular administration of TRD could be used as a "pharmacological scalpel" to remove the inflamed synovium. Our data confirmed anti-inflammatory and anti-proliferative properties of TRD in all experimental models encouraging further studies which should evaluate its therapeutic potential in RA.*

Key words

Taurolidine, taurine, synoviocytes, rheumatoid arthritis, collagen induced arthritis (CIA).

Janusz Marcinkiewicz, Piotr Głuszko, Ewa Kontny, Beata Kwaśny-Krochin, Małogrzata Bobek, Wojciech Wierzchowski, Marta Ciszek, Włodzimierz Maśliński.

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Please address correspondence to: Prof. Janusz Marcinkiewicz, Department of Immunology, Jagiellonian University Medical College, 18 Czysta Street, 31-121 Cracow, Poland.
E-mail: mmmarcin@cyf-kr.edu.pl

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Introduction

Taurolidin (TRD) (bis (1,1-dioxoperhydro-1,2,4-thiabiazin-4-yl)methane) is a derivative of the amino acid taurine. TRD is degraded *in vivo* into three biologically active breakdown products: taurultam, taurinamide and taurine (1). TRD was originally designed as a chemotherapeutic agent owing to its bactericidal and anti-lipopolysaccharide properties (2). Moreover, it has been shown that TRD exerts anti-inflammatory effects by decreasing the secretion of proinflammatory mediators (10). Reflecting these activities, TRD was mainly used in the treatment of patients with peritonitis and in patients with sepsis (3, 4).

In recent years anti-angiogenic and anti–tumor properties of TRD have been demonstrated (5). TRD inhibits cell proliferation and induces apoptosis of various tumor cell lines *in vitro* (6, 7). Subsequent to these experimental observations, encouraging clinical results were seen after intravenous administration of TRD in patients with gastrointestinal and nervous system tumors (6).

Taurine, the third breakdown product of TRD, does not share these activities. However, taurine chloramine (TauCl) and taurine bromamine (TauBr), the physiological products of reaction between taurine and HOCl/HOBr, exert bactericidal and anti-inflammatory properties (8, 9). In our previous studies we have shown that all these taurine derivatives (TRD, TauCl and TauBr), but not taurine itself, can down-regulate inflammation (10). Also, a number of other studies demonstrated that TauCl attenuates the development of arthritis in animal models (11, 12). Interestingly, TauCl inhibited the proliferation of fibroblast-like synoviocytes isolated from rheumatoid patients (RA FLS) (13, 14). Since the reaction of TRD with HOCl results in formation of TauCl, one may speculate that TRD at a site of inflammation, including inflamed joints, will act in a similar manner as TauCl itself (15, 16).

Therefore, in this study we have addressed the issue whether TRD application *in vivo* will affect the development of arthritis. We have used two animal models, collagen induced arthritis in mice (CIA) and ovalbumin induced arthritis in rabbits (AIA). Moreover, we have tested the effect of TRD on RA FLS proliferation and cytokine production *in vitro*.

Materials and methods

Reagents

Taurolidine (Taurolin® for injections - *Boehringer Ingelheim,* Germany*)*: 2% w/v aqueous isotonic solution of taurolidine (TRD) in 5% polyvinylpyrrolidon (PVP) (*Sigma*, USA), was used as a source of TRD. PVP, the pharmaceutical stabilizer of TRD, was used as a control (placebo). For the *in vitro* study, TRD, which has low molecular weight (284), was isolated from Taurolin and separated from PVP (40 000 MW) by centrifugation (2000 x g, 20 min) on Vivaspin membrane 5000 MW (*Vivascience*, Germany).

Patients

Patients who fulfilled the American College of Rheumatology criteria for the diagnosis of RA (17) were underwent knee synovectomy or joint replacement surgery as a normal part of clinical care. Tissue was processed within 2 hours after removal from the patient.

Cells

Synovial fibroblast cell lines were prepared from synovial samples obtained from RA patients as described previously (13). The cells were seeded into tissue culture flat (*TPP*, Switzerland, 25cm^2) (2.5 x 10^4 cells/cm^2) and cultured overnight in a humidified 5% CO_2 atmosphere at 37°C. The non-adherent cells were washed out. Adherent cells were cultured in a complete medium made of RPMI 1640 medium (*JR Scientific Inc*., Woodland, CA, USA) supplemented with 2mM L-glutamine, 100units/ml penicillin, 100μg/ml streptomycin, 50μg/ml gentamycin, 20mM HEPES buffer and 10% fetal calf serum. Then, at confluence, were passaged into fresh tissue culture flat after trypsin/EDTA (*Sigma*, USA) treatment. RA FLS were used for experiments after 3 to 6 passages. At that time all cells showed fibroblast-like morphology.

Analysis of cytokine production in RA FLS by ELISA

For the cytokine production assay 24-well flat-bottom culture plates (*TPP,* Switzerland) were seeded with $2x10^4$ cells in culture medium RPMI 1640. After 2-3 days of culture, fresh complete medium was added and RA FLS were stimulated with 1ng/ml of interleukin 1β (IL-1β), in the presence of TRD at a concentration of 20-100μM for 24 hours. Thereafter, interleukin 6 (IL-6) and interleukin 8 (IL-8) presence was assessed in culture supernatants and cell lysates. The ELISA for IL-6 and IL-8 was performed as described previously (13).

Human recombinant cytokine standards were from R&D systems. The standard curves were determined in culture medium containing 10% FCS. Optical density was measured at 492nm. The detection limit was 15 pg/ml for IL-6 and 4 pg/ml for IL-8.

RA FLS proliferation assay

Proliferation of RA FLS was evaluated on the basis of ^{3}H-thymidine incorporation into the cells. For the assay, 96-well flat-bottom culture plates (*TPP*, Switzerland) were seeded with $5x\ 10^3$ cells in 0.2 ml of culture medium RPMI 1640 and stimulated with 10 ng/ml platelet-derived growth factor (PDGF) (*PeproTech,* USA) in the presence of TRD at a concentration of 20-100μM for 72 hours.^{3}H-metyl-thymidine (2μCi/ml; *Amersham*, UK) was added 18 hours before termination of the cell cultures. Cells were collected from the culture plates using a cell harvester (*Skatron*, Norway) and radioactivity of the samples was measured using a liquid scintillation counter (*1209 Rack-beta*, Sweden).

Viability of RA FLS

RA FLS (5×10^3 /well) were cultured in the presence of TRD (20 -100 μM). Cytotoxicity of TRD was measured in supernatants after 24h and 72h of incubation by means of LDH activity (lactate dehyrogenase) using LDH assay kit (*Takara Shuzo Co*., Japan).

Mice

Inbred DBA1/J female mice from the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College, Cracow, were used between 8 to 10 weeks of age. All mice were housed 3 per cage in the laboratory room with water and standard diet provided *ad libitum*. The authors were granted permission by the Local Bioethical Committee to use mice in this study.

Induction and evaluation of collagen-induced arthritis (CIA)

Mice were immunized with 200 μg of chicken collagen II (CII) emulsified in complete Freund's adjuvant (CFA; *Sigma-Aldrich*, Germany) by intradermal injection at the base of the tail (primary immunization). On day 21, after the first immunization, the mice were immunized subcutaneously with 100 μg of CII in CFA (booster immunization) (11). Mice were then randomized into two groups. One group received intraperitoneal injections of 1ml of 2% Taurolin (TRD 100 mg/kg) two times weekly in 3 consecutive weeks starting on the 21st day of experiment. Control animals were treated with PVP as a placebo. During the study, mice were examined visually twice a week, for the incidence and severity of arthritis (arthritis index) (18).

Measurement of serum anti-collagen antibody titers

Mice were anaesthetized and bled on days 21 and 42. Serum level of antibody against type II collagen (IgG α CII) was measured using a standard ELISA as described previously (11). The antibody level was expressed in arbitrary ELISA units calculated from IgG αCII titer: 1 Unit = 1/100 titer of IgG specific to native collagen II.

Measurement of MPO activity

On day 42 of experiment MPO activity was measured in periarticular tissue as described before (11). The activity of MPO was calculated from a MPO (*Calbiochem*, USA) standard curve and expressed in units. One unit of MPO activity was defined as that degrading 1μmol of H_2O_2 per minute at room temperature. Each sample was measured in duplicate.

Rabbits

6 female New Zealand rabbits, 2.5 kg of weight each, from the Animal Breeding Unit, Department of Clinical Immunology, Institute of Pediatrics, Cracow, were used in this experiment. All animals were housed one per cage in the laboratory room. The authors were granted permission by the Local Bioethical Committee to use rabbits in this study.

Induction of antigen-induced arthritis (AIA)

Arthritis was evoked using a modified method of Sanchez-Pernaute (19). Rabbits were immunized by two subcutaneous injections (days – 0 and 14) of 5 mg (1 ml) of ovalbumin (OVA; *Sigma-Aldrich,* Germany) emulsified with 1 ml of complete Freund's adjuvant (CFA; *Sigma-Aldrich*, Germany). Monoarticular arthritis of the knee joint was induced five days after the second immunization (the 19th day of experiment) *via* intra-articular injection of 0.5 ml (5 mg) of OVA into the right knee of the animal. The left knee was not injected.

Treatment and evaluation of arthritis in rabbits

On day 19 of experiment, rabbits were randomised into two groups. TRD-treated rabbits received 0.5 ml of 2% Taurolin (TRD + PVP) mixed with 5 mg OVA intra-articularly while control rabbits received 0.5 ml of OVA mixed with PVP (placebo). This treatment was repeated twice a week, during consecutive 4 weeks. Before each injection of TRD, the animals were examined for clinical signs and symptoms of arthritis. The mediolateral joint diameters were measured using a vernier caliper. Three measurements for each knee were taken and the values averaged. The experiment was terminated on day 49, six days after the last intra-articular injection of TRD/PVP + OVA. All rabbits were euthanized, arthritic knees were examined macroscopically and parts of the synovial tissue were removed, placed in buffered formalin and then processed for histological evaluation which was performed blindly by a pathologist.

Measurement of serum SAA
Blood samples were collected from all animals on days 0, 19, 33, 49. The serum level of amyloid A (SAA) was measured using Serum Amyloid A ELISA Kit (*BioSource International,* USA).

Statistical analysis
The non-parametric Mann-Whitney U test was applied to examine differences in amounts of cytokine production, antibody synthesis and in cumulative incidence of arthritis.
P values less than 0.05 were considered to be statistically significant.

Results
Effect of TRD on RA FLS proliferation and cytokine production in vitro
In our experimental set-up *in vitro*, TRD exerted significant cytotoxic effect on RA FLS when applied at concentrations > 100 μM. The effect was time dependent. IC_{50}, a 50% cytotoxic concentration of TRD for the 24 h and 72 h incubation time was 500 μM and 100 μM, respectively (Fig. 1). To determine whether TRD exerted any effect on cell proliferation, TRD and PVP were added to non-stimulated and PDGF-stimulated RA FLS. TRD inhibited spontaneous and PDGF-triggered proliferation of RA FLS, in a dose dependent manner (Fig. 2). Moreover, we have tested the effect of TRD on the production of IL-6 and IL-8 by RA FLS stimulated with IL-1, the primary proinflammatory cytokine. TRD strongly inhibited cytokine-triggered synthesis of IL-6 with IC_{50} of ~80 μM, as shown in Fig. 3. The effect of TRD on IL-8 synthesis was weaker. TRD at non-cytotoxic concentrations, only slightly reduced IL-1 – triggered production of IL-8 (by 20-30%). PVP, on the other hand, did not affect any tested functions of RA FLS.

Effect of TRD on the development of CIA in mice – a systemic treatment
Immunization of DBA mice with collagen II resulted in a wide range of IgG αCII serum concentrations (Fig. 4). As anti-CII antibodies are essential for the development of CIA (20), the serum level of IgG αCII was monitored in each mice individually during the experiment.

Fig. 1. Cytotoxicity of TRD. RA FLS (5 x 10^3/well) were cultured in the presence of TRD (20 -100 μM). Cytotoxicity of TRD is expressed as LDH activity (%) measured in supernatants(black bars-after 24h (n = 16) And striped bars-after 72h (n = 13) of the incubation), *p = 0.01 – 0.0001, n- number of experiments in which FLS from different patients were used.

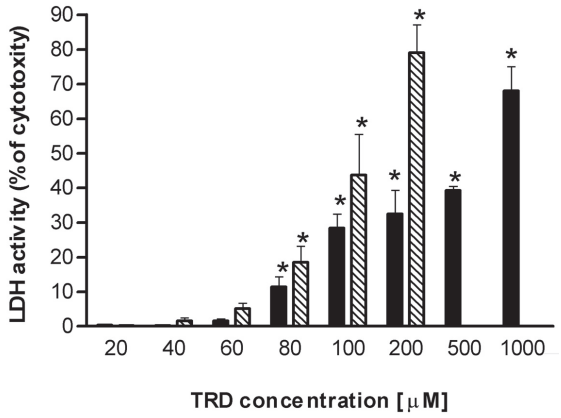
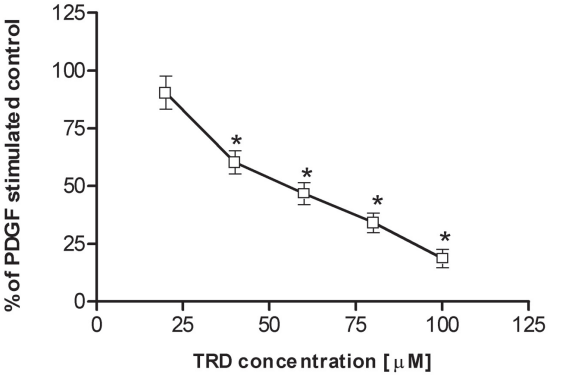


Fig. 2. Effect of TRD on the proliferation of RA FLS.
RA FLS (5 x 10^3/well) stimulated with PDGF were cultured for 72h in the presence of TRD (20–100 μM). Proliferation of the cells was determined by ^{3}H-thymidine intake. Results are expressed as the mean ± SEM from 10 independent experiments. *p = 0.001 – 0.0001 (PDGF treated vs PDGF + TRD treated cells).



The evaluation of CIA development in control mice showed that the severity (incidence, arthritic index, MPO activity in periarticular tissue) of arthritis correlated with the level of IgG αCII (Table I). Mice with high level of IgG αCII antibodies developed severe arthritis, with incidence of 100%. In mice with a low level of IgG αCII antibodies the incidence of CIA was significantly lower but still above 60%. Comparison of all TRD treated mice (n = 13) with control mice (n = 14), independently of IgG αCII serum concentration, shows a strong reduction of CIA incidence by TRD (30%) as compared to (79%) incidence of CIA in PVP treated mice (Fig. 5). However, the effectiveness of TRD was related to the level of collagen specific antibodies achieved after primary immunization (Table I). All mice with high serum level of IgG α CII (> 1000 U) developed severe CIA in both TRD and PVP group (incidence = 100%). On the other hand, TRD, as opposed to PVP, completely abolished the development of CIA (incidence = 0%) in mice with intermediate and low level of IgG αCII (Table I). In addition, mice treated with TRD showed significantly lower final level of serum IgG α CII (day 42) as compared with control mice at that time.

Effect of TRD on the development of AIA in rabbits – a local treatment
The onset of AIA was observed 3 days after the first intra-articular injection of OVA (day 22 of experiment) in all rabbits, as indicated by increased diameters of right knees (mean Δ 5.1mm) (Fig. 6). Since then, until the end of experiment the diameters of swollen joints in rabbits treated with TRD + OVA remained unchanged. In contrast, animals receiving PVP + OVA developed more pronounced oedema of arthritic knees, reaching their maximum on days 27-29 (the additional increase of knee diameter = Δ 3.3- 3.6 mm). Then, the oedema was gradually reduced, especially at the final stage of the experiment (day 42-49) (Fig. 6).
Macroscopic evaluation of the knees of PVP treated rabbits revealed pannus formation and synovial effusion.

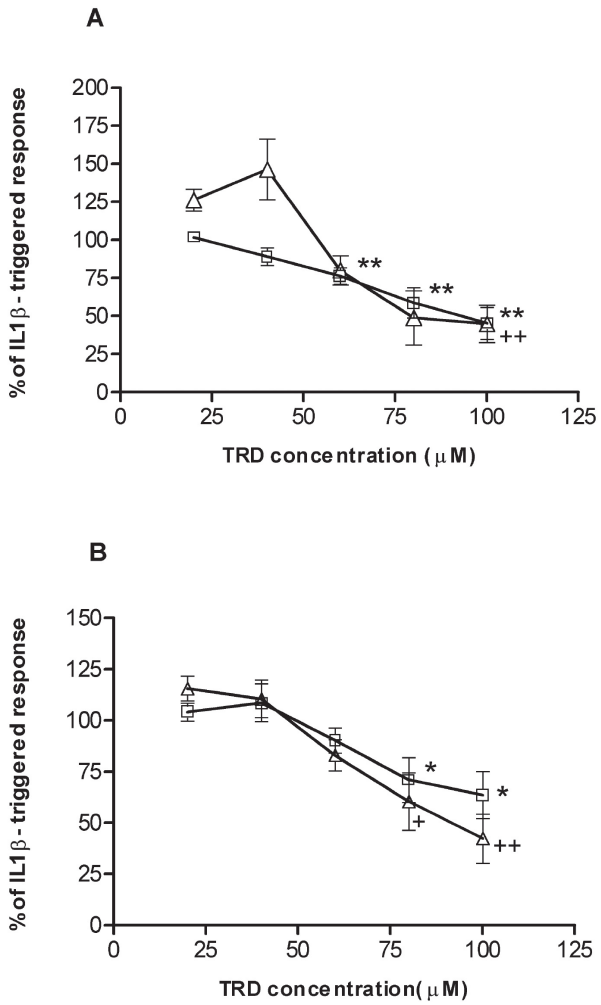


Fig. 3. Effect of TRD on the production of IL-6 and IL-8 by RA FLS.
RA FLS (2 x 10^4 /ml) were stimulated with IL-1β in the presence of TRD (20-100 μM) for 24h. The concentrations of IL-6 (**A**) and IL-8 (**B**) were determined in culture supernatants -❑ and cell lysates -Δ. Results are expressed as the mean ± SEM from at least 7 experiments. In figure (**A**) $^{**}p = 0.002\text{-}0.0002$, $^{++}p = 0.002$, in figure (**B**) $^{*}p = 0.05\text{-}0.01$, $^{+}p = 0.05\text{-}0.01$, $^{++}p = 0.002$.

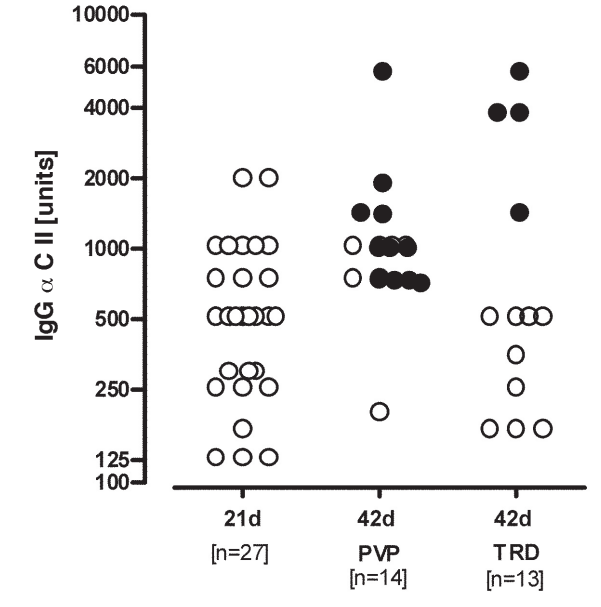


Fig. 4. Humoral response to type II collagen in CIA.
The level of IgG specific to CII in individual mice sera, collected on day of the booster immunization (day 21st) and day 42, was determined by ELISA. Results are expressed in arbitrary ELISA unit (1 U = 1/100 titer of IgG αCII. Each circular symbol represents one mouse, ● – CIA (+) mice, ○ – CIA (-) mice.

Synovial tissue obtained from PVP treated joints showed features of synovitis with synovial lining hyperplasia, characteristic papillary configuration, with lymphocyte and other mononuclear cell infiltration and the presence of some lymphoid follicles (Fig. 7A, B). On the contrary, in TRD treated animals extensive necrosis of synovial membranes (synoviorthesis-like effect) has been observed macroscopically. Necrotic lesions of synovial membranes with lymphocytes, plasmocytes and polymorphonuclear leukocytes infiltration were confirmed in a microscopic examination (Fig. 7 C,D).

The initial level (63-117 ng/ml) of SAA, a systemic feature of inflammation (21), increased substantially after subcutaneous immunization with OVA + CFA (data from day 19), but with high inter-animal variability. SAA concentration reached its maximum on day 33 and eventually dropped down to the range of initial values in all rabbit sera (Fig. 6). Owing to a small number of animals (the decision of Bioethical Committee) and variations of SAA serum concentration, the differences observed between TRD treated and control animals cannot be clearly interpreted. However, all TRD treated rabbits show smaller increase of SAA serum level then control animals when the maximum concentration of SAA (day 33) was compared with the concentration observed on day 19.

Discussion

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease manifested by a progressive synovial joint inflammation and altered humoral and cell-mediated immune response. Overgrowth of fibroblast-like synoviocytes (RA FLS) as well as their secretion of an impressive array of proinflammatory mediators play a crucial role in the pathogenesis of RA (22), therefore removal of the inflammatory synovium should ameliorate inflammation and arrest the progress of joint destruction (23). Indeed, potential strategies for the treatment of RA are currently focused on reducing the production of inflammatory cytokines and inducing apoptosis of synoviocytes (23, 24). Such pharmacologically induced apoptosis of RA FLS might play a similar role to classical synovectomy but without any surgical tissue damage.

Previously, we have suggested that TauCl, a product of reaction between taurine and HOCl, is a promising candidate for treatment of arthritis (11).

Table I. Final evaluation of the CIA development in mice treated with TRD and PVP.

[a]Experimental groups			Final evaluation of the CIA development (day 42)			
Serum IgG αCII[d] (U) Day 21	Treatment Days 21-42	Number of mice	Incidence (%)	[b]Arthritis index	[b]MPO activity U/mg protein	[c]Serum IgG αCII[d] (U)
"high" (> 1000)	TRD	4	4/4 (100)	7.25 ± 2.5	0.112 +/- 0.09	3712 ± 2043
	PVP	3	3/3 (100)	9.3 ± 3.2	0.130 ± 0.08	2900 ± 2600
"intermediate" (250-1000)	TRD	5	0/5 (0)	0	0	*550 ± 268
	PVP	8	6/8 (75)	6.9 ± 4.7	0.064 ± 0.05	1088 ± 307
"low" (< 250)	TRD	4	0/5 (0)	0	0	*224 ± 146
	PVP	3	2/3 (66)	3.5 ± 0.5	0.023 ± 0.02	661 ± 379

[a]Mice were twice immunized with CII + CFA (primary immunization – day 0, booster immunization – day 21st). On day 21 mice were divided into three groups: mice with "high", "intermediate" and "low" serum concentration of IgG αCII. Animals from each group were randomized and subsequently treated either with TRD or PVP (placebo) as described in *Methods*.
[b]Results are expressed as the mean ± SE calculated from CIA (+) mice, CIA (-) mice show arthritic index = 0 and MPO activity = 0.
[c]Results from all mice/group were taken and show as the mean ± SE.
[d]IgG αCII - 1U = 1/100 titer of IgG specific to collagen.
*$p < 0.05$ (TRD vs PVP).

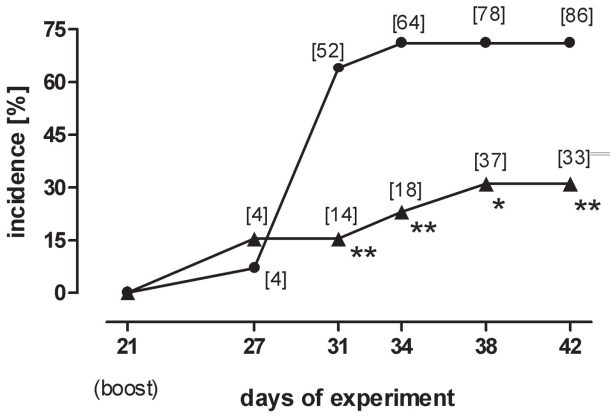


Fig. 5. Effect of TRD treatment on the development of CIA.
Mice were immunized with CII in CFA twice (the primary immunization was followed after 21 days by the booster immunization), as described in Methods. TRD (1.0 ml of 2% Taurolin) was injected intraperitoneally twice a week, starting 1 day after the booster immunization till day 42. Control mice received PVP as placebo. During the study all animals were visually examined twice a week. CIA incidence (the percentages of arthritic animals) at the indicated times are given. Severity of arthritis is shown in brackets as the arthritis index summarized from all CIA positive animals. (x) – arthritis index ,● - placebo (n =14), ▲ - TRD (n =13).
*$p < 0.05$, **$p < 0.01$ (Incidence TRD vs PVP).

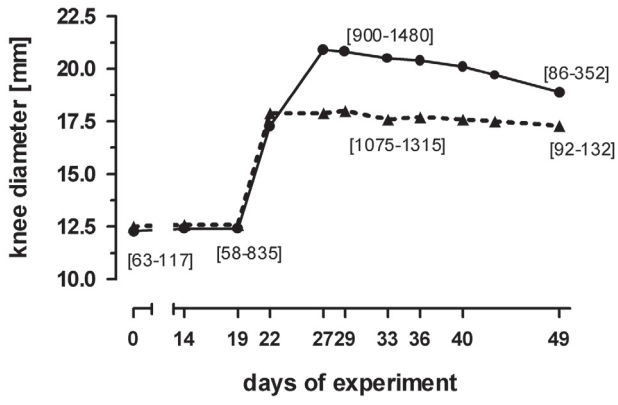


Fig. 6. Evaluation of the AIA development in rabbits treated with TRD and PVP.
Six rabbits were immunized with OVA in CFA (0 and day 14). To induce and maintain AIA, animals were injected into right knee with OVA (days: 19, 22, 27, 29, 33, 36, 40, 44). At the same time three rabbits received TRD (0.5 ml of 2% Taurolin) and three rabbits received PVP, as described in Methods. Results are shown as the right knee diameters measured at the indicated times (three measurements were taken and the values averaged). ▲ –TRD, ● - PVP (placebo), (x-y) – the range of serum level of SAA (ng/ml) is given in brackets.

TauCl inhibited the production of proinflammatory cytokines by synoviocytes, and, more importantly, inhibited proliferation of RA FLS *in vitro* (13, 14). However, *in vivo* administration of TauCl in animal models of arthritis (CIA) has a therapeutic effect only after local, intra-articular injection (12). On the other hand, systemic administration of TauCl prior to the onset of arthritis significantly reduced the incidence of CIA (11). Only recently, we have shown that TRD, another taurine derivative, effectively reduced the development of zymosan-induced peritonitis in mice (10). Moreover, comparative study *in vitro* showed similar anti-inflammatory effects of TRD and TauCl, but mediated by different mechanisms (10).

In our present study we have examined the effect of TRD on the development of arthritis after local and systemic administration. We have also examined *in vitro* the influence of TRD on cytokine production and proliferation of synoviocytes taken from RA inflamed joints. These studies clearly indicate that TRD, at non-cytotoxic concentrations, exerts anti-proliferative and anti-inflammatory properties. These results are in agreement with other reports which show anti-tumor activity of TRD exerted by inhibition of cell proliferation of many tumor cell lines (5-7).

The results from the *in vivo* study, clearly indicate that local intra-articular administration of TRD (Taurolin) results in a marked decrease of AIA severity in rabbits exerting a synoviorthesis–like effect in arthritic synovium. In this experimental treatment Taurolin (2% solution of TRD)

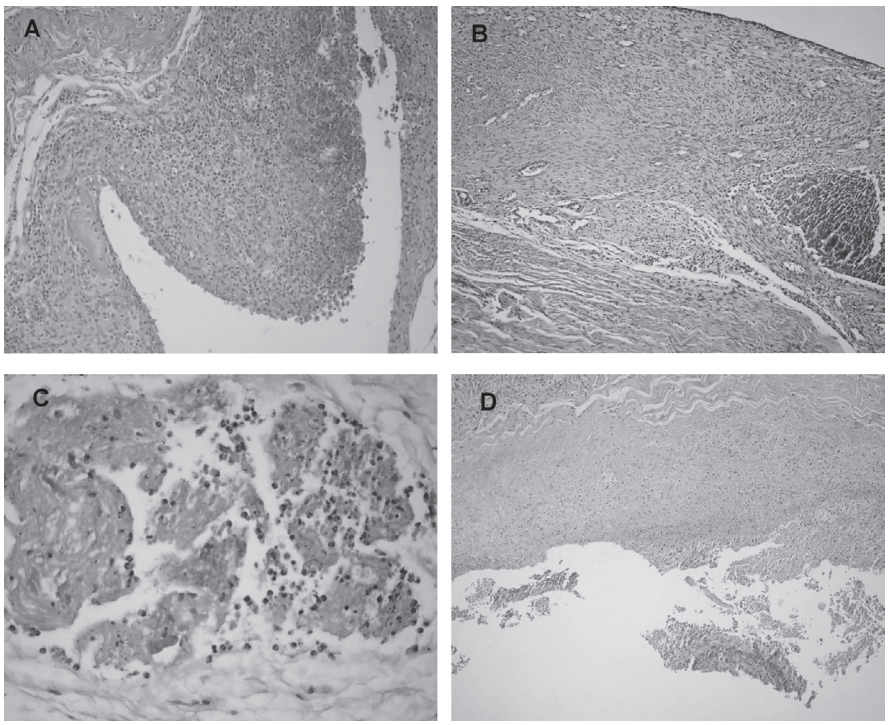


Fig. 7. Histopathology of AIA in rabbits treated with PVP and TRD.
(A, B) - PVP –treated synovium with papillary configuration and mononuclear cell infiltration of the subsynovium. (C, D) - TRD –treated synovium showing signs of inflammation and areas of necrotic lesions (hematoxylin and eosin stained, magnification 165 x in A, magnification 80 x in B, D and 330 x in C).

was used in a dose equivalent to that used in peritoneal Taurolidine lavage in children with localized peritonitis (3). Histopathology of the arthritic tissues revealed extensive necrotic lesions in synovium. As TRD shows *in vitro* cytotoxic effect on RA FLS, confirming its effectiveness against proliferating cells, we think that in our models hyperproliferating synoviocytes seems to be the most probable target for TRD. However, the mechanisms of TRD activity remain unclear. Further studies are necessary to determine which of TRD breakdown products exerts its anti-proliferative activities.

In contrast to AIA, in collagen induced arthritis (CIA) TRD was administered intraperitoneally. Our data indicate that systemic injections of TRD before the onset of arthritis significantly reduce the incidence of CIA except for highly immunized animals. The results from placebo treated mice confirmed that the incidence and severity of CIA correlated with the serum level of anti-collagen IgG antibodies (IgG αCII). Mice which did not develop CIA showed statistically lower serum concentration of IgG αCII than arthritic mice. It may be explained by the fact that collagen type II (CII) is a relevant joint-specific autoantigen in the pathogenesis of rheumatoid arthritis (RA) (25, 26). Moreover, the reduction of serum levels of anti-collagen antibodies was observed in various types of therapies of CIA and was associated with decreased severity and incidence of arthritis (27, 28). Interestingly, none of the mice with the serum concentrations of IgG αCII below 1000 Units developed CIA during the treatment with TRD while placebo treated mice developed CIA even with much lower concentrations of IgG α CII (< 250 Units). It may suggest that the reduction of incidence of CIA by systemic administration of TRD is the result of its effect on IgG αCII production. However, further studies are necessary to evaluate this hypothesis. It would be crucial to demonstrate the suppressive effect of TRD on the production of IgG2a αCII. Meaningful data are available to suggest that a Th1 environment is required for the induction of a proinflammatory anti-collagen T cell response and induction of IgG2a isotype, which are essential for the development of CIA (29, 30).

In conclusion, this study confirmed anti-inflammatory and anti-proliferative properties of TRD. The anti-arthritic effects of TRD demonstrated in our animal models of arthritis encourages further studies to confirm its therapeutic potential in various forms of arthritis, including RA. Results from AIA and from *in vitro* RA FLS studies suggest that intra-articular administration of TRD could be used as a "pharmacological scalpel" to remove inflamed synovium. In our opinion, despite the promising beneficial effect on CIA after systemic administration, TRD is more suited to be used as a local agent, owing to its activity *in vivo*.

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