Original Article

Leflunomide Derivate FK 778 in Accelerated Renal Injury in Transgenic Rat

(ischaemia/reperfusion injury / FK 778 / leflunomide / transgenic rat / chronic allograft nephropathy / hypertension)

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Abstract. Renal ischaemia/reperfusion (I/R) injury and hypertension represent major alloantigen-independent risk factors contributing to the development of chronic allograft nephropathy. In a model of accelerated major histocompatibility complex-independent renal injury, we evaluated the effect of leflunomide derivate – FK778 – on the progression of accelerated nephropathy. Thirty-six uninephrectomized hypertensive transgenic (m-REN-2)-27 rats received a clip on renal pedicle for 45 minutes. Animals were treated with FK778 3 mg/kg/day (I/R 3 mg, N = 12), 10 mg/kg/day (I/R 10 mg, N = 12) or placebo (N = 12) via gavage for 16 weeks. Eighteen animals were sham-operated and treated with FK778 3 mg/kg/day (sham 3 mg, N = 6), 10 mg/kg/day (sham 10 mg, N = 6) or were untreated (sham, N = 6). Proteinuria and blood pressure were evaluated throughout and the kidneys were harvested for morphological and immunohistochemical analysis at the end of the experiment. At week 16, rats with I/R injury and FK778 treatment had lower proteinuria compared with placebo-treated rats (I/R 3 mg: 48.42 ± 26.16, I/R 10 mg 27.28 ± 21.86 vs. Placebo: 70.13 ± 50.19 mg/day, P < 0.05). The untreated sham group exhibited lower proteinuria compared with FK778-treated sham groups (Sham 3 mg: 24.23 ± 10.89; Sham 10 mg: 17.37 ± 4.13; Sham: 14.23 ± 1.18) There was no difference in glomerulosclerosis and interstitial fibrosis among the treated groups. In the untreated animals the rate of interstitial fibrosis decline reached statistical significance (Placebo vs. Sham: 1.125 ± 0.641 % vs. 0.250 ± 0.500 %, P < 0.05). There was higher $CD5^+$ leukocyte infiltration in the placebo-treated group. FK778-treated rats displayed amelioration of some changes induced by the I/R injury. Our observation also suggests potential nephrotoxicity of FK778.

Introduction

Despite the indisputable advancements in transplantation medicine, chronic changes characterized as interstitial fibrosis and tubular atrophy (IF/TA; previously termed as chronic allograft nephropathy – CAN) remain the leading cause of late renal graft loss. Recently, the alloantigen-dependent and independent factors, especially chronic activation of the renin-angiotensin-aldosterone system, ischaemia/reperfusion (I/R) injury and deviations in the immune system regulation have been determined to play a pivotal role in the induction and progression of renal graft dysfunction (De Greef et al., 2002).

To study the long-term consequences of ischaemia/reperfusion injury, we used the well-defined animal model of progressive nephropathy. This experimental model of angiotensin II-dependent hypertension is represented by the (m-REN-2)-27 hypertensive transgenic rat (TGR) that harbours the mouse gene for renin *ren-2* in the genome of the normotensive Hannover Sprague-Dawley (HanSD) rat. As a result of this gene transfection, the TGR develops severe hypertension, proteinuria and glomerulosclerosis (Viklicky et al., 2004; Rajnoch et al., 2005).

FK778, a synthetic derivate of leflunomide, belongs to the new group of immunosuppressants – isoxasole derivates. The immunosuppressive effect lies in the in-

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Abbreviations: CAN – chronic allograft nephropathy, HanSD rat – Hannover Sprague-Dawley rat, ICAM-1 – intracellular adhesion molecule 1, IF/TA – interstitial fibrosis and tubular atrophy, I/R – ischaemia/reperfusion, MHC – major histocompatibility complex, MMF – mycophenolate mofetil, PAS – periodic acid-Schiff, TGF-β – transforming growth factor β, TGR – transgenic rat, VCAM-1 – vascular adhesion molecule 1.

hibition of a key enzyme of pyrimidine synthesis – mitochondrial dihydroorotate-dehydrogenase – and in inhibition of tyrosine-kinase activity associated with cytokines and growth factor receptors (Schrepfer et al., 2006). The mechanism of FK778 action results in direct inhibition of T and B lymphocytes, decrease of serum allo-specific immunoglobulin (IgG, IgM) molecule production and significantly decreased expression of adhesion molecules (intracellular adhesion molecule 1 (ICAM-1); vascular adhesion molecule 1 (VCAM-1)) that are essential for transendothelial leukocyte migration and cytokine synthesis (Schrepfer et al., 2005).

The animal experiments exhibited an unambiguous effect of FK778 therapy on the development of histological changes accompanying chronic allograft changes (IF/TA), including reduction of mononuclear cell infiltration ($CD4^+$ lymphocytes, $CD8^+$ lymphocytes, ED-1 macrophages) and transforming growth factor β (TGF-β) graft expression. The benefit of short-term therapy with FK778 has already been proved; however, the benefit of long-term application (more than 20 days) in comparison with adverse effects still remains unclear (Deuse et al., 2003).

Since immune cells have been implicated in the course of chronic progressive nephropathies, we hypothesized that immunosuppression may slow down the progression of accelerated nephropathy. Thus, we evaluated the effect of dihydroorotate-dehydrogenase inhibition by FK778 in our model of accelerated renal injury.

Material and Methods

The experiments were performed in 12-week-old male hypertensive heterozygous TGRs. The rats were housed in climate-controlled conditions with a 12-h light/dark cycle and had free access to standard diet and drinking water. The animals were handled according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), and the local authorities approved the experimental protocol.

Drugs

FK778 was obtained as a generous gift from Astellas Pharma (Munich, Germany). A suspension of FK778 in distilled water was prepared fresh daily before use. The dose of 3 mg/kg/day or 10 mg/kg/day of FK778 was administered daily by oral gavage. The treatment was initiated on the first day after surgery and terminated after 16 weeks.

Surgery

The surgery was performed under general anaesthesia induced by sufentanil (20 μg/kg, Sufenta forte, Janssen-Cilag, Beerse, Belgium) and azaperone (1 mg/kg, Stresnil, Janssen) administered intramuscularly. Through a midline incision, the left renal artery and vein were isolated, occluded with a microvascular clamp for 45 min and the left kidney was cooled with ice. Clamps were removed and blood flow was re-established. The right kidney was removed at the time of surgery. The abdominal incision was then re-sutured. In the case of sham operation, only the abdominal cavity was opened and after 45 min closed again. The animals with any apparent signs of unsuccessful operation were discarded.

Experimental protocol

The rats were assigned into six groups. In group A (I/R-FK778 3 mg/kg/day, N = 12) rats underwent I/R injury and were treated from the first day with FK778 at the dose of 3 mg/kg/day; in group B (I/R-FK778 10 mg/kg/day, N = 12) rats underwent I/R injury and were treated with FK778 at the dose of 10 mg/kg/day. In group C (Sham-FK778 3 mg/kg/day, N = 6) rats were sham-operated and treated from the first day with FK778 at the dose of 3 mg/kg/day; in group D (Sham-FK778 10 mg/kg/day, N = 6) rats were sham-operated and treated with FK778 at the dose of 10 mg/kg/day. In group E (Sham, N = 6), rats were sham-operated and not treated, and in group F (Placebo, N = 12) rats underwent I/R and received placebo. The experiments were terminated at 16 weeks.

Harvesting

In the early postoperative period, two animals died in the 3 mg/kg/day FK778-treated group, one in the 10 mg/kg/day FK778-treated group and two in the placebo-treated group that underwent I/R injury. During the experiment, one rat died in the 3 mg/kg/day FK778-treated group, and two in the placebo-treated groups after I/R injury. In sham-operated rats, two animals of each group died in the early postoperative period. The most common causes of death were severe pneumonia or pulmonary oedema. At the end of the study, the kidneys were harvested, blood from aorta collected, and the rats were sacrificed under general anaesthesia. The representative portions of kidneys were fixed in either formalin for histological evaluation or isopentan for immunohistochemical evaluation.

Functional measurements

The rats were weighed once per week. In monthly periods, the systolic blood pressure was measured and the animals were placed into metabolic cages. The systolic blood pressure was assessed in non-anaesthetized rats by indirect tail pletysmography (indirect rat-tail blood pressure system, Harvard Apparatus, Edenbridge, UK). The rats were placed in metabolic cages once per four weeks and 24-h urine samples were collected, cooled and analysed quantitatively for proteinuria (by nephelometry, Boehringer, Mannheim, Germany), creatinine (Jaffe method, Unicon, Biotech, Menlo Park, CA), urea (thiosemicarbazide, Unicon, Biotech), sodium and potassium by standard autoanalyser methods (Hitachi Automatic Analyzer, Boehringer).

Morphological analysis

Formalin-fixed and paraffin-embedded tissue samples were stained by haematoxylin-eosin to assess the grade of tubular atrophy and tissue cellular infiltration

and by the periodic acid-Schiff (PAS) reaction to determine the degree of glomerulosclerosis, interstitial fibrosis, inflammatory cell infiltrate, and vascular changes. Tissue sections were coded and examined in a blind fashion by light microscopy.

Glomerulosclerosis was defined as an increase in mesangial matrix and/or collapse of capillaries. PAS-positive staining of the sclerotic lesions, adhesion of obsolent segment of Bowman's capsule and hyaline entrapment were observed. All glomeruli were counted in each section and the percentage of sclerosis was calculated. Tubulo-interstitial injury was defined as interstitial fibrosis, tubular atrophy and dilatation accompanied by interstitial inflammatory infiltrate. Renal structural damage was graded semi-quantitatively on a scale of 0 to 3+ for interstitial fibrosis, tubular atrophy and vascular changes according to cellular infiltration, tubulopathy and arterial intimal fibroplasias by Banff 97 criteria, and the sum of scores (0 to 9+) was calculated for each sample.

Immunohistochemical analysis – detection of CD 4, CD 5, CD 8, ED-1

Immunohistochemistry was performed with 5 µm-thick frozen sections. Samples were fixed in acetone and air-dried. Endogenous biotin was blocked using Biotin blocking system (Vector Laboratories, Burlingame, CA). The tissues were then pre-incubated with 10% horse serum (Vector Laboratories) for 30 min to prevent unspecific binding. Primary antibody (anti CD4, CD5, CD8, ED 1 monoclonal antibody, Chemicon Int, Ltd., Dorchester, UK) was applied for 60 min in appropriate dilution. On negative control slides the step with monoclonal antibody was omitted. Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 20 min. Detection of monoclonal antibodies was done using biotinylated anti-mouse IgG (H+L) (Vector Laboratories) diluted in 1% bovine serum albumin with 2% rat serum, 50x, for 30 min. The specimens were then incubated with R.T.U. Vectastain Elite ABC Reagent (Vector Laboratories) for 30 min. Finally, specimens were stained with 3,3'-diaminobenzidine (DakoCytomation, Copenhagen, Denmark) for 5 min and counterstained with Harris's haematoxylin before they were embedded in Entellan (both from Merck, Darmstadt, Germany).

Statistical analysis

All data are expressed as mean ± SD. Comparison between treated/untreated hypertensive groups was performed by Kruskal-Wallis test with χ^2 distribution. Statistical significance was accepted at $P < 0.05$.

Results

Functional measurements

At the beginning of the experiment we did not observe any differences in proteinuria among the groups. After 16 weeks, the rats that underwent I/R injury and were treated with FK778 exhibited significantly lower proteinuria compared with rats treated with placebo (Group A vs. Group F: 48.42 ± 26.16 vs. 70.13 ± 50.19; Group B vs. Group F: 27.28 ± 21.86 vs. 70.13 ± 50.19; $P < 0.05$). Surprisingly, in the untreated sham group, there was a tendency towards lower proteinuria compared with sham groups treated with FK778 (Group C: 24.23 ± 10.89; Group D: 17.37 ± 4.13; Group E: 14.23 ± 1.18) (Fig. 1).

During the course of the experiment we did not observe any statistically significant differences in systolic

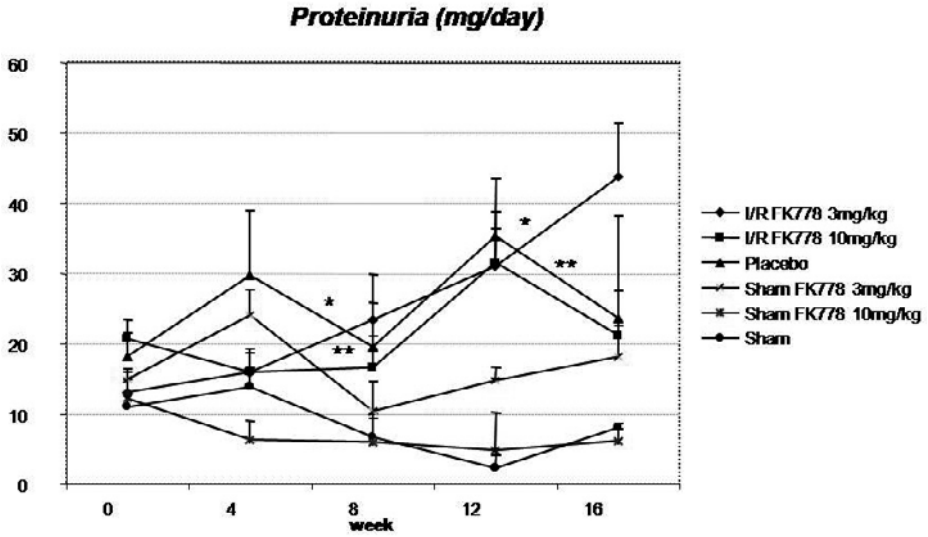

Fig. 1. Development of the proteinuria experiment in 16-week follow-up.
During 16 weeks after ischaemia/reperfusion injury we observed that FK778-treated rats exhibited lower 24-h urine protein excretion compared with placebo-treated rats. FK778-treated rats had higher 24-h proteinuria than untreated sham rats. Values represent mean ± SEM. *$P < 0.05$ I/R-FK778 3 mg/kg vs. placebo, **$P < 0.05$ I/R-FK778 10 mg/kg vs. placebo.

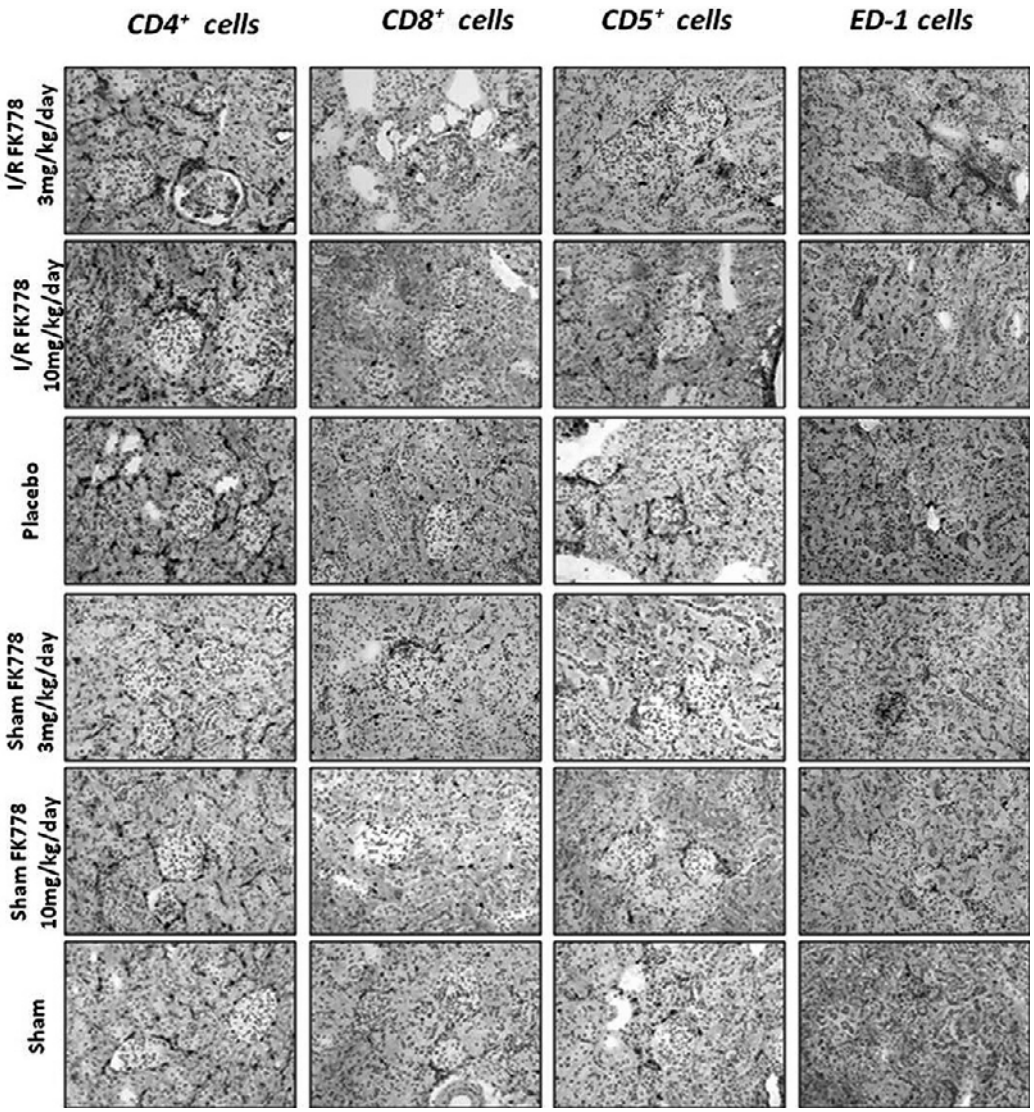

Fig. 2. No differences in cellular infiltration, except for $CD5^+$ cells, were observed.

Table 1. Functional and histological parameters in the analysed animal groups
The FK778-treated rats after I/R injury exhibited higher urea and creatinine clearances than the placebo-treated animals. The FK778-treated sham animals had significantly lower creatinine and urea clearances compared to the untreated sham group. There were no differences in blood pressure development during 16-week follow-up – the animals remained hypertensive independently of the therapy. Despite the tendency to reduction of glomerulosclerosis in FK778-treated groups that underwent I/R injury compared to placebo, only the difference in interstitial fibrosis between both groups reached statistical significance.

	Urea clearance (ml/s/100 g b.w.)	Creatinine clearance (ml/s/100 g b.w.)	Blood pressure (mmHg)	Glomerulosclerosis (%)	Interstitial fibrosis (grade 0–3)
Group A	0.0409 ± 0.0214	0.1404 ± 0.1193	206.00 ± 23.70	12.5070 ± 8.3965**	0.7500 ± 0.6614
Group B	0.0540 ± 0.0188	0.1011 ± 0.0725	202.33 ± 23.30	7.9950 ± 5.9353	0.4545 ± 0.4979
Group C	0.0364 ± 0.0095	0.0791 ± 0.0235	226.25 ± 8.84	9.6200 ± 3.0218	0.5000 ± 0.5000
Group D	0.0391 ± 0.0099	0.0769 ± 0.0316	197.00 ± 22.53	6.9010 ± 2.8515	0.7500 ± 0.4330
Group E	0.0671 ± 0.0301[a]	0.1661 ± 0.0878[b]	202.75 ± 12.09	9.9725 ± 4.6728	0.2500 ± 0.4330[c]
Group F	0.0370 ± 0.0149[a]	0.0744 ± 0.0328[b]	201.38 ± 16.62	14.8940 ± 7.0384	1.1250 ± 0.5995[c]

[a] $P < 0.05$ Placebo vs. Sham; [b] $P < 0.05$ Placebo vs. Sham; [c] $P < 0.05$ Placebo vs. Sham

blood pressure among the groups. All animals remained hypertensive independent of the therapy (Fig. 2).

The analysis of renal function after 16-week follow-up revealed the animals treated with FK778 3 mg/kg/day to have similar creatinine and urea clearances as the animals treated with 10 mg/kg/day FK778 and placebo in both sham groups and groups that experienced I/R injury; however, statistically significant differences in renal

function parameters were found only between untreated groups (creatinine clearance: Group F vs. Group E: 0.074 ± 0.035 vs. 0.166 ± 0.101 ml/s/100 g b.w., $P < 0.05$; urea clearance: Group F vs. Group E: 0.037 ± 0.016 vs. 0.671 ± 0.035 ml/s/100 g b.w., $P < 0.05$) (Table 1).

Morphological analysis

Morphological features represent a mixture of various grades of glomerulosclerosis, vascular changes, interstitial fibrosis and inflammatory infiltration.

Compared with the placebo-treated group, the groups that underwent I/R injury and were treated with FK778 exhibited a trend towards lower extent of glomerulosclerosis, but without statistical significance (Group A vs. Group F: 12.51 ± 8.40 % vs. 14.90 ± 7.09 %; Group B vs. Group F: 7.99 ± 5.94 % vs. 14.90 ± 7.09 %). In sham groups, we did not observe any differences in the extent of glomerulosclerosis. The rate of interstitial fibrosis was significantly decreased in animals treated with a daily dose of 10 mg/kg FK778, but statistical significance was reached only in the untreated groups (Group F vs. Group E: 1.125 ± 0.641 % vs. 0.250 ± 0.500 %, $P < 0.05$). The morphological changes observed in placebo- and FK778-treated groups correlated with proteinuria (Table 1).

Surprisingly, we did not observe any differences in $CD4^+$, $CD8^+$ and ED-1 cell infiltration among groups except for higher grade of $CD5^+$ cells in the placebo-treated group that underwent I/R (Fig. 2).

Discussion

In the present animal model of accelerated nephropathy we evaluated the effect of leflunomide derivate FK778, an inhibitor of dihydroorotate-dehydrogenase, the key enzyme of proliferation and differentiation of leukocytes and monocytes/macrophages.

At 16 weeks after I/R injury we observed differences in proteinuria in the tested animals. The rats that underwent I/R injury and were treated with FK778 exhibited lower proteinuria compared with placebo-treated animals after I/R injury. FK778-treated sham rats had higher proteinuria than untreated sham rats. These results are not related with the functional parameters. Both sham groups and groups after I/R injury treated with 3 mg/kg FK778 have shown similar urea and creatinine clearances as animals treated with 10 mg/kg FK778. The apparent reduction of urea and creatinine clearance was observed only in placebo-treated rats compared to sham rats. These observations might be explained by the protective role of FK778 in the case of I/R insult. On the other hand, in case of uninjured tissue, FK778 might display nephrotoxic effects.

The morphological findings corresponded with proteinuria. Conversely, sham groups treated with FK778 have shown more accented glomerulosclerotic and fibrotic tissue remodelling in comparison to sham groups without treatment. This might suggest the FK778 to be potent in slowing down the progression of renal dysfunction and injury. Nevertheless, in the naïve rats without ischaemic injury (but with hypertension), the FK778 therapy was associated with nephrotoxicity.

Recent studies have reported effects of a leflunomide derivate on the course of chronic transplant changes – interstitial fibrosis and tubular atrophy (Deuse et al., 2004; Lutz et al., 2007). Surprisingly, we have not observed significant differences in inflammative infiltration (except for $CD5^+$ cells). However, recently published papers confirmed the capability of FK778 to reduce the extent of both fibrotic changes and cellular infiltration ($CD4^+$, $CD8^+$, ED-1 cells) in renal graft (Rintala et al., 2006). The possible reason for our differing result (effect on inflammative infiltrate) might lie in the difference in evaluation time and in the used therapeutic dose. The above-mentioned studies evaluated the effect of FK778 at the daily doses of 5–20 mg/kg on the extent of post-ischaemic changes already 10–12 weeks after transplantation. It is possible that at 16 weeks after I/R injury, nephrotoxic rather than therapeutic effects of FK778 dominate, depending on the applied dose. This corresponds with the observation that FK778 at the daily dose of 2.5 mg/kg was ineffective while at 40 mg/kg, the dose was toxic (Pan et al., 2003; Fitzsimmons and First, 2004; Schrepfer et al., 2005).

In the present study, like in our previous experiment with mycophenolate mofetil (MMF), we have observed excessive activation of the immune system (Bloudickova et al., 2006). The therapy with both immunosuppressive agents led to significant reduction of glomerulosclerosis and interstitial fibrosis after I/R injury. In different ways, both MMF and FK778 have a potent inhibitory effect on B-cell proliferation and immunoglobulin formation; moreover, MMF suppresses the ongoing IgG response (Allison and Eugui, 2005). Concerning T cells, MMF markedly decreased the infiltration of CD4–, CD8– lymphocytes and macrophages, whereas leflunomide derivate reduced only $CD5^-$ leukocytes. Species specificity of T- and B-cell sensitivity to FK778 has been observed, based on different species affinities for isoxazole derivates by the enzyme dihydroorotate-dehydrogenase (Vu et al., 2003).

The applied dose reflects the possible therapeutic effects of FK778. Although the animals treated with a higher daily dose (10 mg/kg) exhibited clear reduction of morphological changes similar to sham animals, their functional parameters were poorer than those of the groups treated with a daily dose of 3 mg/kg. Moreover, altered proteinuria, both creatinine and urea clearances, support the hypothesis about the nephrotoxic effect of FK778.

In conclusion, specific reversible dihydroorotate-dehydrogenase inhibitor FK778 ameliorated, to some extent, the accelerated injury induced by ischaemia/reperfusion in rats. The mechanism of the observed nephrotoxicity, however, remains to be further elucidated.

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