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Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection

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Abstract

Leflunomide has been shown to be very effective in preventing and curing several autoimmune animal diseases. Further, this agent is as effective as cyclosporin A in preventing the rejection of skin and kidney transplants in rats. Preliminary results from patients suffering from severe cases of rheumatoid arthritis demonstrated that clinical and immunological parameters could be improved with leflunomide therapy. Mode of action studies revealed that this substance antagonizes the proliferation inducing activity of several cytokines and is cytostatic for certain cell types. In this light, we could show that tyrosine phosphorylation of the RR-SRC peptide substrate and the autophosphorylation of the epidermal growth factor (EGF) receptor were, dose dependently, inhibited by leflunomide. EGF activates the intrinsic tyrosine kinase of its receptor, which stimulates the phosphorylation of a variety of peptides, the amino acid residue in all cases is tyrosine. These results indicate that much of leflunomide's activity could be due to the inhibition of tyrosine-kinase(s), which is an important general mechanism for the proliferation of various cell types. Thus, leflunomide, which is effective against autoimmune diseases and reactions leading to graft rejection, would seem to have a mode of action separating it from known immunosuppressive drugs.

Introduction

Leflunomide (HWA 486) an isoxazol derivative with antiphlogistic and novel immunomodulating properties, would seem to be a universal drug to combat autoimmune disorders [1–10]. Although the pharmacological profile of this substance has recently been reviewed [1], so much more data has been generated that a new review is warranted. Here we will briefly cover the already published results and present some new and preliminary data dealing with leflunomide's effects in animal models of autoimmunity and organ transplantation, as well as some of our most recent *in vitro* findings concerning the mode of action of its active metabolite, A771726. Further, very preliminary clinical data concerning leflunomide's effects on the immune response of patients with rheumatoid arthritis will be presented.

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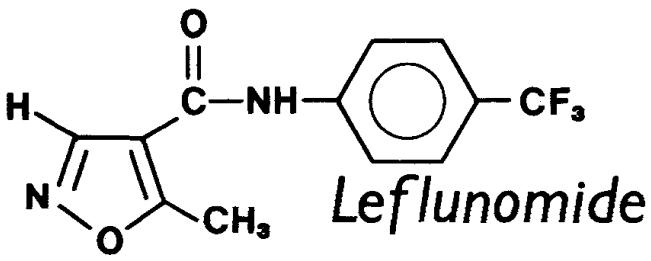

Autoimmune animal studies

Leflunomide inhibits the adjuvant disease of rats

Studies dealing with effects of leflunomide on the adjuvant arthritic disorder, of Lewis rats, offered us the initial clues that leflunomide may have anti-inflammatory and immunomodulating properties. Using the "standardized arthritic assay" described by Peper et al. [11] to differentiate between non-steroidal anti-inflammatory drugs and immunosuppressive agents, we found that not only was leflunomide able to arrest the development of adjuvant arthritis, but, unlike immunosuppressive agents that were considered to be exclusively active in this assay, it could restore the diminished mitogen induced lymphocyte response of the diseased animals [2]. Further, although effective in this assay, to our surprise, leflunomide did not demonstrate immunosuppressive activity, at least concerning the ex-vivo response of lymphocytes to mitogens in healthy rats [2].

These results were independently confirmed by Pasternak et al. [6], as well as Hambleton and McMahon [8]. Pasternak found further that leflunomide significantly reduced edema, fibrinogen levels, and erythrocyte sedimentation rates. The antiarthritic effects of this agent were more sustained than those observed after cyclosporin A (CSA) therapy. Whereas both leflunomide and CSA could reduce the delayed type hypersensitivity (DTH) response to mycobacterial antigen on day 9 followed by a rebound to an enhanced DTH response on day 21, only leflunomide was able to restore the suppressed mitogenic response of splenocytes to phytohemagglutinin (PHA), a T-cell mitogen. Hambleton and McMahan confirmed the suppressive effects of leflunomide and CSA on the early (day 10) DTH-reaction, with no effect, on this reaction, when tested on day 15. Similar results were observed when these animals were treated with prednisolone (PRED), whereas neither indomethacin nor tiaprofenic acid influenced the DTH reaction.

Leflunomide arrests murine systemic lupus erythematosus (SLE)-like disease of MRL/lpr-mice

SLE is an autoimmune disease that affects multiple body organs and is characterized by the development of certain types of self antigens. Primarily, the antibodies formed against double-stranded DNA (dsDNA), the most prevalent in this ailment, complex together and, with complement, deposit in the small blood vessels, leading to widespread vasculitis. MRL Mpf lpr/lpr (MRL/lpr)-mice spontaneously develop a severe disease with many symptoms very similar to human SLE, i.e. hypergammaglobulinaemia, and glomerulonephritis [3, 12, 13]. These mice may equally well serve as a model for human rheumatoid arthritis, especially considering the articular involvement, such as swelling of the pawns, pannus formation, proliferation of synovial tissue, degradation of articular cartilage, and the presence of circulating rheumatoid factor (RF) [3, 12–14].

Treatment of MRL/lpr mice with leflunomide dose dependently arrested disease progression and prevented the development of glomerulonephritis [3, 4]. This was due to the suppression of circulating immune complexes (Table 1), which was a direct result of the greatly lowered autoantibody formation, such as those to dsDNA or to immunoglobulins (RF) [3, 4]. Further, the tremendous number of lymphocytes which accumulate in the lymphnodes and spleens of MRL/lpr-mice could be greatly suppressed, depressing the amount of the double negative T-cells, i.e. T-lymphocytes possessing neither CD4 (T-helper cell phenotype) nor CD8 (T-suppressor cell phenotype) differential antigens (Table 1). At the same time the ratio of CD4/CD8-T-cells, which is greatly increased in these mice, was restored to normal values. These effects were also observed after treatment of these animals with CSA (Table 1). Furthermore, leflunomide therapy could restore not only the suppressed proliferative response of lymphocytes to T-cell mitogens (PHA and concanavalin A (Con A)), but also the depressed activity of macrophages to phorbolmyristenacetate (PMA) [4].

The disease inhiting effects of leflunomide were not limited to prophylactic activity [7]. MRL/lpr mice that had elevated levels of protein in their urine

Table 1
Effects of drugs on MRL/lpr-disease.

Mouse strain	Drug	Dose mg/kg/day	No. double neg. T-cells [1]	CD4/CD8 ratio	Proteinuria mg/mouse	Circulating immune complexes [2]
C3H	none	0	30	2.5	<0.1	0.1
MRL/lpr	none	0	1000	7.0	1.4	2.0
MRL/lpr	HWA486	35	100	2.0	0.1	0.5
MRL/lpr	CSA	100	50	1.9	1.7	0.5

Therapy was initiated when the animals were 10 weeks old and terminated when the animals were 22 weeks of age. The data given was obtained 6 weeks after the last drug application.
[1] Double negative T-cell are those whithout CD4 or CD8 differential antigens (double negative/lymphnode).
[2] Data is given as OD at a serum titer of 1/3200.

were aministered, for 10 weeks, either leflunomide, CSA, or prednisolone (PRED). At the end of the medication period, 90% of the animals receiving leflunomide were still alive, whereas 50% of the non-treated control mice and only 40% of the CSA or PRED medicated animals survived. Although all of the surviving mice had normal urine-protein levels, only the leflunomide medicated rodents had significantly reduced levels of RF and autoantibodies to dsDNA. CSA and PRED treatment resulted in amplified titers of autoantibody to dsDNA. It would appear that leflunomide is better suited to combat the established affliction of MRL/lpr mice than either CSA or PRED.
As to the question about what happens when leflunomide therapy is terminated after the animals have become "healthy", we have published results offering a good answer [1]. We treated MRL/lpr mice with either 35 mg/kg leflunomide or 20 mg/kg azathioprine, starting when the animals were 10 weeks old. After 9 weeks (19 weeks of age), the therapy was discontinued and the disease development followed. The progression of the ailment in animals given azothioprine could be slowed down, but, even before the therapy was terminated, the symptoms of the disorder advanced to the same level as that of non-treated MRL/lpr-mice. Leflunomide therapy, on the other hand, not only prevented the appearance of symptoms, but 20 weeks after the treatment was ended, no signs of the illness could be detected [1], although they did slowly appear somewhat later.

Leflunomide therapy prevents paralysis in experimental allergic encephalomyelitis (EAE)

EAE is a T cell mediated, neurologic autoimmune disease that develops in susceptible animals following sensitization with either spinal cord homogenate, or myelin basic protein [15]. Although the induction of EAE is essentially due to cellular immune reactions [16], there is increasing evidence for an additional role of humoral factors in the pathogenesis of this illness [17]. EAE in animals is considered to be an appropriate model for multiple sclerosis (MS) in man [18]. In Lewis rats, clinical EAE is characterized by the development of transient hindquarter paralysis [1].
Studying the effects of leflunomide on the prevention of paralysis in an acute form of EAE in Lewis rats, we found that this agent was as effective as CY [1]. Yet, contrary to the effects of CY, splenocytes from animals treated with leflunomide responded normally to T and B cell mitogens [1].

Leflunomide prevents organ specific nephritic diseases

Examples of leflunomide's effects on organ-specific autoimmunity have been very recently reported from two independent laboratories using two different animal models of nephritic disorders. Thoenes et al. [10] demonstrated that this agent is very effective in preventing experimental tubulointerstitial nephritis (TIN) in rats. TIN is induced by immunizing animals with either homologous or heterologous tubular basement membranes (TBM) in Freund's complete adjuvants. In rats, TIN commences at about 10 days after TBM (from sheep) stimulation, leading to serious damage to the kidney cortex and decreased kidney function [19]. In the above mentioned study [10], it was found that leflunomide was just as effective as CSA, but more efficacious than PRED, naproxen, or indomethacin in preventing disease development. Regarding the inhibition of autoantibody

formation to TIN, leflunomide was much more effective than the other drugs tested.
Using an antibasement membrane antibody induced glomerulonephritis in rats, Ogawa et al. [9] could show that an oral dose of 2 mg/kg/d leflunomide resulted in significant decrease in total urinary protein, plasma cholesterol and fibrogen, as well as decreased incidences of fibrin, IgG and C_3 deposits. This was the case for both preventive (two days before disease induction and ending on day 20) as well as curative (five days after induction and ending on day 20) drug therapy.

Effects of leflunomide on inflammatory and allergic reactions

Concerning antiallergic activity, we have observed that leflunomide effectively inhibits the edema formation in the skin of guinea pigs sensitized with specific IgE (passive cutaneous anaphylaxis test) [1]. Further, this drug was as effective as phenylbutazone in inhibiting the inflammatory reaction induced by carrageenan [1].

Leflunomide suppresses reactions leading to organ transplantation rejection and graft versus host diseases

Effects of leflunomide on mice undergoing a chronic graft-versus-host (CGVH) reaction

The intravenous injection of a mixture of parental splenocytes into healthy inbred F_1-mice results in graft-versus-host (GVH) induced immune abnormalities. This is due to T-lymphocytes in the donor inoculum that recognize the major histocompatibility alloantigens (murine H_2-antigens) expressed by the F_1-animals. The host F_1 T-cells are genetically unable to recognize antigens of the parental donor as foreign, thus the response involves only donor recognition of host and non host recognition of donor. The ensuing immune abnormalities depend on the parental and F_1 strain combinations used. For example, the inoculation of C57BL/6 spleen cells into (C57BL/6 × DBA/2) F_1-mice, further referred to as DF_1-mice, leads to the development of an acute GVH (AGVH))-disease resulting in profound immunodeficiency, anemia, hypogammaglobulinemia, the appearance of suppressor cells [20] and the development of cytotoxic T-lymphocytes (CTL) specific to BDF_1-alloantigens [21]. In contrast, inoculation of DBA/2 cells into BDF_1-mice results in a chronic GVH (CGVH)-reaction in which lymphoid hyperplasia, autoantibody production, immune complex glomerulonephritis [21, 22] and the failure to form CTL to BDF_1-alloantigens [20], i.e., an illness resembling human systemic Lupus erythematosus (SLE).
First, we studied the effects of leflunomide on the chronic graft versus host (CGVH) disease of mice, i.e. animals undergoing a disease displaying symptoms very similar to SLE. Comparing the protective effects of this agent to those of CY, PRED, and indomethacin, we found that when therapy was started 4 weeks after disease induction (shortly before the first appearance of proteinuria), only indomethacin was ineffective in inhibiting the SLE-like symptoms [5]. Curiously, although PRED could prevent the development of glomerulonephritis and thus proteinuria, it did not inhibit the deposition of immune complexes on the glomeruli [5]. This may be due to the mode of action of steroids, which have been reported to inhibit complement, as well as the production of interleukin-1 (IL-1) [1]. Interestingly, although leflunomide is not a cytotoxic agent, as is CY [23], and to some extent PRED, the splenomegaly of the CGVH-diseased mice was dose-dependently inhibited after therapy with this agent [5].
As the case in both adjuvant- and MRL/lpr-diseased animals, mice undergoing a CGVH-reaction have significantly suppressed lymphocyte responses to T cell mitogens (Con A and PHA). Treatment with leflunomide restored these responses, whereas neither indomethacin nor PRED displayed any positive effects. Depending on the dose, CY partly restored or inhibited these mitogen induced responses of T cells [5, 23].

Prevention of skin and kidney graft rejection by leflunomide

In the prevention of reactions leading to transplant rejection, leflunomide initially seemed to be completely ineffectual [1]. Although successful in preventing the chronic graft-versus-host (CGVH)-disease [5], this agent was first reported not to have any protective activity in the runting illness brought on by an acute GVH reaction [1]. Due to the results obtained from the effects of this agent on the murine CGVH-disease, and considering that all studies we [1–7] and others [6, 8, 9] had

Table 2
Effects of drug therapy on allogenic kidney transplantation.

Transplant	*n*	Drug	Dosage (mg/kg/d)	Plasma-creatine levels on day (mg/dl ± SD)					Survival rate (d ± SD)
				8	32	40	50	60	
Syngenic	7	none	0	0.7 ± 1.2	0.6 ± 0.4	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.3	> 60
Allogenic	6	none	0	7.0 ± 16.1					8.0 ± 0.3
Allogenic	12	HWA486	5 (po)	0.7 ± 0.7	0.7 ± 0.8	0.7 ± 0.6	0.7 ± 0.3	0.7 ± 0.3	> 60
Allogenic	13	HWA486	10 (po)	0.7 ± 0.7	0.7 ± 0.7	0.7 ± 0.7	0.7 ± 0.8	0.7 ± 0.8	> 60
Allogenic	13	CSA	10 (po)	0.8 ± 0.4	0.7 ± 0.5	0.7 ± 0.8	0.7 ± 0.9	0.7 ± 0.5	> 60
Allogenic	7	AZA	5 (iv)	7.9 ± 17.4					8 ± 0
Allogenic	7	PRED	5 (iv)	6.9 ± 4.6					7.9 ± 0.6

Lewis rats were transplanted with either syngenic (Lewis) or allogenic (BN) kidneys. Drug therapy was initiated on day −1 and terminated on day 30 as indicated. AZA = azathioprine; CSA = cyclosporin A; PRED = prednisolone; HWA486 = leflunomide; po = per os; iv = interveinous. From Küchle et al. [24].

Table 3
Effects of leflunomide therapy on allogenic skin transplantation.

Transplant	*n*	Dosage (mg/kg/d)	Graft survival time (d/SD)
DA→LEWIS	10	0.0	10.5 ± 1.1
DA→LEWIS	10	2.5	19.7 ± 1.9
DA→LEWIS	10	5.0	23.7 ± 2.0
DA→LEWIS	10	10.0	27.0 ± 1.1
DA→LEWIS	10	20.0	29.1 ± 1.8
LEWIS→FISHER	10	0.0	16.2 ± 1.0
LEWIS→FISHER	10	2.5	22.6 ± 2.2
LEWIS→FISHER	10	5.0	25.9 ± 2.1
LEWIS→FISHER	10	10.0	28.9 ± 1.7
LEWIS→FISHER	10	20.0	33.8 ± 2.8

Rats were treated from day 1 to 10 with leflunomide (per os) after tail skin was transplanted. From Küchle, et al. [24].

conducted demonstrated that leflunomide was just as effective as CSA in the therapy of various autoimmune disorders, we reasoned that this drug must also be efficient in preventing transplantation rejection reactions.

Using Lewis rats (RT 1l), as host animals, kidneys from BN rats were transplanted. In the untreated rats, these allografts were rejected within eight days, whereas treatment with leflunomide, for 30 days, prolonged the graft and thus the animal survival of all of these animals for the duration of the experiment (more than 60 days) (Table 2). Following the serum-creatinine levels we could determine that the transplanted kidneys functioned normally (Table 2), and the histological studies revealed virtually no signs of chronic rejection [24]. The results we obtained from CSA therapy were very similar to those observed after leflunomide, whereas, in our experiment, neither azathioprine nor prednisolone offered any protection (Table 2).

Looking further, we found that leflunomide was not only efficacious in suppressing kidney but also skin rejection reactions in rats. Using two different strain combinations for our studies, DA/Lewis (MHC and non-MHC different) and Lewis/Fisher (non-MHC different), tail skin from the donor animals was grafted to the hosts. Therapy with leflunomide was started one day after transplantation and terminated on day ten. Using this protocol, a dose dependent depression of the rejection time could be observed in both transplant combinations (Table 3). With this protocol, i.e. starting drug application a day after exposure to foreign antigen, CSA is not efficient, at least in our hands. This is because CSA is much better suited to suppress primary reactions, before they are initiated, and is much less efficient in inhibiting ongoing immune reactions [1, 7, 25]. This indicates a much different mode of action of leflunomide than that of CSA.

Studies concerning the mode of action of leflunomide

Ex vivo and in vivo studies

For a long time, we felt that leflunomide did not have any or very little influence on T-cells. It seemed that this drug asserted its effects chiefly on B-cells, or perhaps T-cell products mediating B-cell activity. This was based on our findings that

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