Inflamm Res 44: 317–322 (1995)

1023-3830/95/080317-06 $ 1.50+0.20/0

Leflunomide interferes with pyrimidine nucleotide biosynthesis

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Received 23 January 1995; returned for revision 20 April 1995; accepted by G. Carter 9 May 1995

Abstract. Leflunomide is an anti-inflammatory and immunosuppressive agent which blocks proliferation of transformed cells and mitogen stimulated normal lymphocytes but does not block T cell signalling mechanisms at antiproliferative concentrations. These properties are consistent with a mechanism involving interference with nucleotide metabolism. Leflunomide had anti-proliferative activity against all cells tested here. The anti-proliferative activities could be reversed by addition of uridine or cytidine to the cultures although some species and cellular differences were observed. Purine nucleosides had no effect. Measurements of nucleotide pools in a human T cell line and mitogen stimulated rat spleen cells treated with leflunomide showed that leflunomide preferentially reduces pyrimidine nucleotide levels. These results indicate that inhibition of pyrimidine biosynthesis is responsible for the anti-proliferative effects of leflunomide.

Key words: Leflunomide – Purine nucleotide – Dihydroorotate dehydrogenase – DHOD – T cell

Introduction

Leflunomide is an immunosuppressive molecule which ameliorates autoimmune diseases and inflammatory processes in several animal models [1]. Recently reported animal studies in which leflunomide showed efficacy include xenogeneic transplants, cardiac and skin allografts, progressive polyarthritis, corneal transplants, autoimmune uveitis and experimental allergic encephalomyelitis [2-10]. In the rat skin allograft model, transferable tolerance was induced by leflunomide treatment [5]. In a clinical dose-ranging study leflunomide arrested disease in patients with active rheumatoid arthritis and was well tolerated [11].

The mechanism of action of leflunomide remains elusive. It does not have NSAID properties [12]. Leflunomide only partially reduces autocrine cytokine production and IL-2 receptor expression and its effects are not reversed by exogenous cytokine [2, 13, 14]. It has been suggested that leflunomide inhibits tyrosine kinase activity [15, 16]. However, leflunomide is as effective against stimuli such as phorbol ester and ionomycin that bypass initial signalling events as it is against receptor triggered stimuli in lymphocyte proliferation assays and it is fully effective when added as late as eight hours after mitogens [14]. These results suggest that leflunomide inhibits events which initiate progression through cell cycle that occur downstream of initial signalling. Leflunomide is also anti-proliferative towards transformed cells of non-lymphoid as well as lymphoid origin [1, 14]. This suggests that leflunomide may be similar to the immunosuppressant rapamycin which binds endogenous immunophilins and consequently interferes with a component of the p70-S6 kinase signalling pathway which is responsible for entry into the cell cycle [17]. These observations suggest that leflunomide is also similar to agents such as brequinar or mycophenolic acid which disrupt nucleotide metabolism and block DNA replication [18, 19]. Here we have investigated an alternative mechanism of action for leflunomide, namely whether it interferes with nucleotide metabolism.

Methods

Reagents

The active metabolite of leflunomide, RS61980-000 (or A771726), was synthesized by M. Rider (Syntex). In vivo, leflunomide is converted to the active metabolite upon first pass through the system (data not shown). Hence, all in vitro experiments reported here utilize the metabolite but it will be referred as leflunomide. Brequinar was synthesized by J. P. Dunn (Syntex). PHA, ConA and all nucleotide intermediates were obtained from Sigma (St. Louis, MO) and were prepared as sterile filtered stock solutions in media. Nucleotide standards were from Boehringer-Mannheim (Indianapolis, IN).

Cells and culture conditions

The mouse B lymphoma A20-1.11 (ATCC, Rockville, MD) and

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mouse B cell hybridoma 3DO-18.3 [20] were cultured in RPMI-1640 (Whittaker, Walkersville, MD) with 5% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 10μM β-mercaptoethanol. The rat PC12 pheochromocytoma cell subclone ACH2 [21] and the human T lymphoblastoid line Jurkat (ATCC, Rockville, MD) were cultured in media without 10μM β-mercaptoethanol. These cells were cultured at 10^4 cells/200 μl media/well in 96 well plates for the proliferation assays. CCRF.CEM cells were grown in RPMI-1640 with 10% FBS and penicillin, streptomycin and L-glutamine supplements.

Rat spleen cells were isolated from female CD rats (100-150 gms, Charles River, Wilmington, MA). Mouse spleen cells were isolated from female Balb/C mice (15–18 gms, Charles River). Rodent lymphocytes were isolated by centrifugation over a Ficoll gradient to remove red cells. Rodent cells were cultured in 96 well plates at 2×10^5 cells/well in a 200 μl volume in a medium consisting of RPMI-1640 (Whittaker, Walkersville, MD) with 5% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 10 μM β-mercaptoethanol. Nucleotide intermediates were added from sterile stock solutions prepared in media. Cells were stimulated with an optimally mitogenic concentration of ConA (2 μg/ml) for 3–4 days.

Human whole blood was collected from normal donors, centrifuged for 15 min at 300×g, and the platelet-rich-plasma removed. Blood was reconstituted to its original volume with Dulbecco's phosphate buffered saline (PBS) without Ca^{++} and Mg^{++}. Human peripheral blood mononuclear cells (HPBMC) were separated using a layer of Ficoll-Hypaque underneath a 1 to 4 dilution of reconsitituted blood and PBS. Preparations were centrifuged at 500×g for 30 min at room temperature. HPBMC were harvested and washed several times with PBS without Ca^{2+} and Mg^{2+}. HPBMC were cultured in RPMI-1640 with 5% pooled human AB serum. Nucleotide intermediates were added prior to stimuli from sterile stock solutions prepared in media. Cells were stimulated with an optimally mitogenic concentration of PHA (10 μg/ml) for 3–4 days.

MTT assay of viability and proliferation

The density of cells in the culture wells was assayed with the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) as described [22]. Culture media (100 μl) was removed from each well, MTT (10 μl of 5 mg/ml stock) was added to the remaining 100 μl and the culture was returned to the incubator for 4 hours. Acid isopropanol (150 μl 0.04 N HCl/isopropanol) was added to the culture to dissolve the purple formazan product. Specific absorption by the reduced product was determined on a 96 well absorption spectrophotometer using a test wavelength of 570 nm and a reference wavelength of 650 nm. Accumulation and reduction of MTT within the mitochondria is linearly related to the number of cells in the culture well.

Nucleotide pool measurements

Cell samples were harvested, counted, washed with ice cold PBS, and extracted with 12% cold trichloroacetic acid and adjusted to pH 5–6 with freon/octylamine (7.8/2.2 ratio). After pre-filtering through a 0.22μ nylon filter the samples were analyzed by HPLC using a Partisil 10 SAX column (Whatman, Clifton, NJ). Nucleotides were eluted with a buffer of 0.4 M ammonium phosphate containing 5% acetonitrile, pH 3.25. Elution times were calibrated with nucleotide standards. Dynamax software (Rainin, Emeryville, CA) was used to quantitate the nucleotides (pmol/10^6 viable cells) by comparison with a standard curve generated for each nucleotide. The percent inhibition (leflunomide treated vs untreated) was then calculated.

Results

ACH2, a subclone of the rat pheochromocytoma cell line PC-12 [21], the mouse B cell lymphoma A20-1.11, the

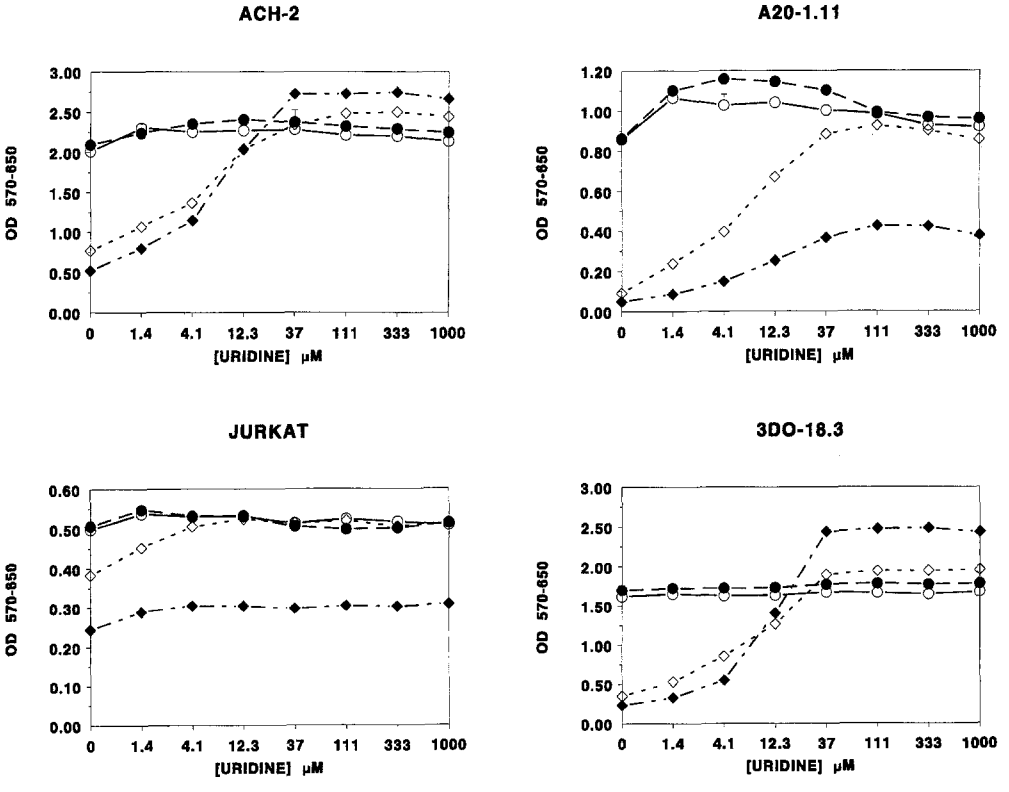

Fig. 1. Uridine blocks the anti-proliferative activity of leflunomide in cell lines. The indicated cell lines were cultured for three days in the absence (○) or presence of 0.5 μM (●), 5 μM (◇) or 50 μM (◆) leflunomide. The indicated concentrations of uridine were added to the cultures at the initiation of culture. Cell density in the cultures was assessed by the MTT assay. Data points are the means of duplicate cultures with the range indicated by error bars which are typically smaller than the data point symbols. DMSO controls for drug had no effect. The experiment was repeated once with similar results.

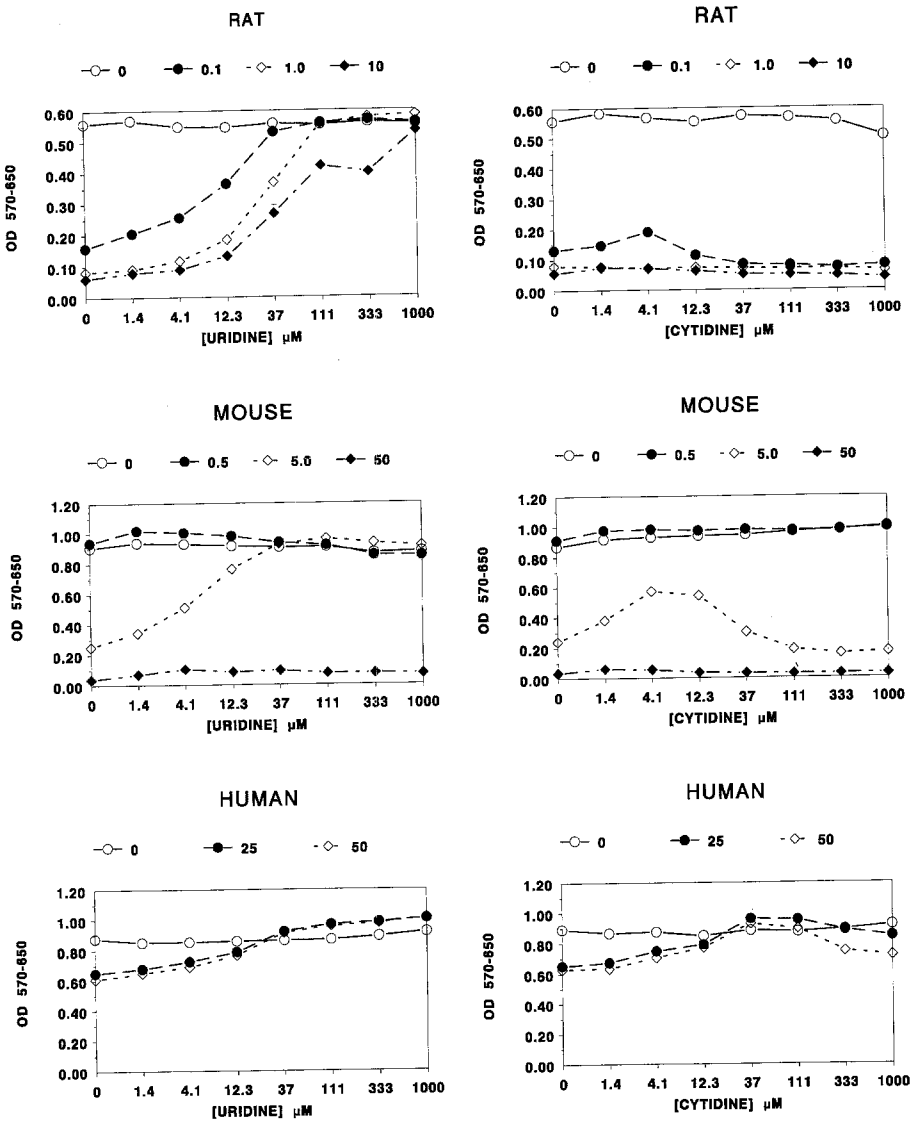

Fig. 2. Uridine and cytidine can antagonize the anti-proliferative activity of leflunomide in normal mitogen stimulated lymphoid preparations. Spleen cell preparations from rats and mice were stimulated with Con A (2 μg/ml). Human peripheral blood mononuclear cells were stimulated with PHA (10 μg/ml). The indicated concentrations of leflunomide (μM) and either uridine or cytidine were added to the cultures just prior to addition of the stimulus. Cell density was assessed with MTT after 3 days. Data points are the means of duplicate cultures with the range indicated by error bars. DMSO controls for drug had no effect. The experiment was repeated once with similar results.

human T lymphoblast Jurkat and the mouse B cell hybridoma 3DO-18.3 were cultured for three days with or without leflunomide (Fig. 1). Inspection by phase contrast microscopy showed that all of the lines proliferated considerably, consistent with observed doubling times of 24 hours or less. In leflunomide treated cultures there was clear microscopic evidence of a reduction in cell numbers with no microscopic evidence of drug toxicity except in the A20-1.11 cultures treated with 50 μM leflunomide. In the latter case some cellular debris was observed. Proliferation of all of the cells was sensitive to 5 μM or less of leflunomide and Jurkat was the least sensitive. When increasing concentrations of uridine were added to the cultures simultaneously with a constant concentration of leflunomide the anti-proliferative effects of leflunomide were overcome by the nucleoside. There were differences in the responses of the cells to uridine. With ACH2 and 3DO-18.3 an enhanced proliferation was caused by high concentrations of uridine and this was only observed if leflunomide was present. The anti-proliferative effect of 50 μM leflunomide on ACH2 and 3DO-18.3 was completely blocked by 37 μM uridine, but the effect of 50 μM leflunomide on A20-1.11 and Jurkat could not be inhibited even with 1 mM uridine. However, the anti-proliferative effect of 5 μm leflunomide on all of these cells was completely blocked by 100 μM uridine or less. Similar experiments were performed with cytidine but up to 1 mM cytidine could only partially block the anti-proliferative effects of leflunomide (not shown). When used at the same concentrations as uridine or cytidine, guanosine was unable to block the activity of leflunomide (not shown).

Table 1. CCRF.CEM cells were cultured with or without the indicated concentrations of leflunomide or brequinar for 18 hours. Cells were harvested, nucleotide levels were determined and the percent reduction in nucleotide levels caused by the drugs as compared to control cells was determined.

Drug [μM]	Percent Inhibition				
	CTP	UTP	ATP	GTP	N
leflunomide [100]	73±4*	84±3	−21±20	2±14	3
leflunomide [10]	51±17	58±22	−19±35	7±20	3
brequinar [10]	76	86	60	-25	1

*values are the mean ± standard deviation.

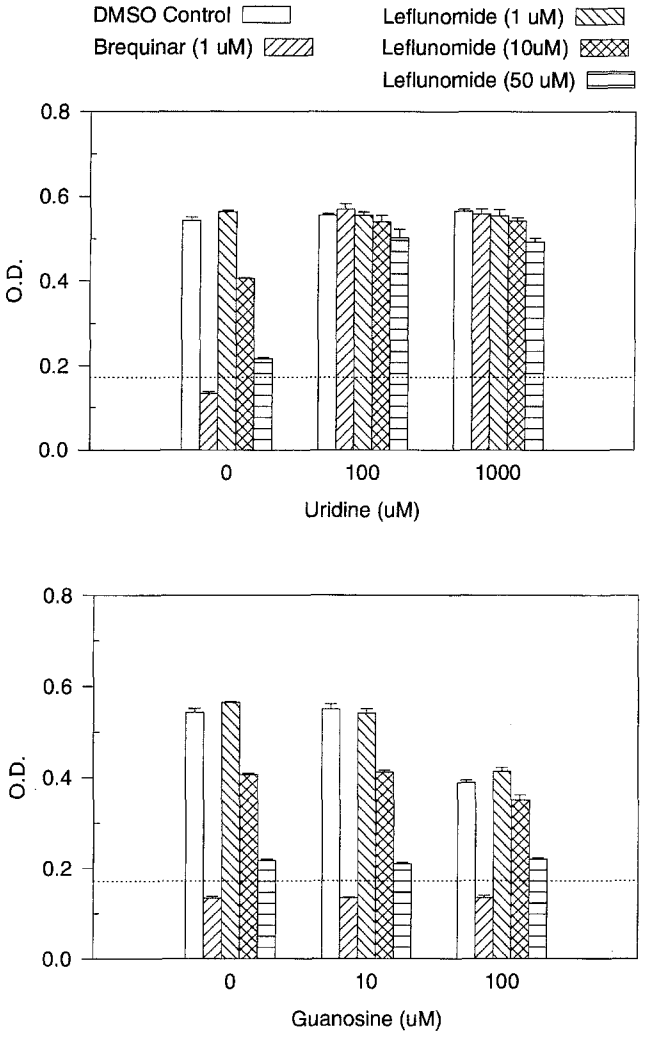

Fig. 3. The anti-proliferative activities of leflunomide and brequinar can be reversed by pyrimidine but not purine nucleosides. Human peripheral blood mononuclear cells were stimulated with PHA (10 μg/ml). Leflunomide (1 μM, 10 μM, 50 μM) or brequinar (1 μM) and uridine (0 μM, 100 μM, 1000 μM) or guanosine (0 μM, 10 μM, 100 μM) were added just prior to the stimulus. As a control a volume of DMSO was added to one culture to raise the DMSO concentration to equal that of the highest dilution used to deliver compound. Cell density was assayed with MTT after 4 days. The dashed line indicates the MTT signal of control unstimulated cells. Data are the mean +/−SD from triplicate samples. Similar results were obtained in three experiments.

The ability of uridine to block the anti-proliferative effect of leflunomide on normal lymphocyte proliferation was studied with mitogen stimulated rat, mouse and human cells (Figs. 2 and 3). As indicated previously [1, 14], when normal rat lymphocytes are stimulated by mitogen they are more sensitive to leflunomide than human or mouse cells. The effects of all concentrations of leflunomide on the rat cells could be blocked by uridine. Similar results were observed with mitogen stimulated mouse spleen cells, although the effects of the highest concentration of leflunomide on the mouse cells was unaffected by as much as 1 mM uridine. This was due to toxicity of 50 μM leflunomide that was apparent microscopically. As with the cell lines, cytidine had only partial effects against leflunomide in the rat and mouse cell assays. Uridine and cytidine completely blocked the effects of leflunomide in mitogen stimulated human peripheral blood mononuclear cells (HPBMC), although an excess of cytidine was ineffective. Similar experiments with HPBMC and guanosine (Fig. 3) or adenosine (not shown) demonstrated that purine nucleosides can not block the anti-proliferative activity of leflunomide. Purines were also ineffective in mouse and rat cells (not shown). Brequinar, which inhibits pyrimidine biosynthesis by inhibiting dihydroorotate dehydrogenase, was included as a control. As predicted, brequinar's activity was reversed by uridine but not by guanosine.

Table 2. ConA stimulated rat spleen cells were cultured with or without the indicated concentrations of leflunomide for 18 hours. Cells were harvested, nucleotide levels were determined and the percent reduction in nucleotide levels caused by the drugs as compared to control cells was determined.

Drug [μM]	Percent inhibition			
	CTP	UTP	ATP	GTP
leflunomide [10]	19.4	38.1	12.5	10.3
leflunomide [1]	18.3	34.5	4	6.8
brequinar [0.1]	10.1	15.2	−3.6	−8.5

Direct measurements of nucleotide pools were performed on samples of the human T lymphoblast CCRF.CEM after eighteen hours of culture with leflunomide. After 18 hours with 100 μM or 10 μM leflunomide the numbers of CCRF.CEM cells in these cultures were reduced by 48% and 12% respectively without overt toxicity. Nor was toxicity observed after 68 hours of culture. The results of three experiments are summarized in Table 1. Leflunomide at 100 μM or 10 μM caused significant reduction in the levels of CTP and UTP but did not affect the levels of purine nucleotides. Brequinar, an inhibitor of pyrimidine biosynthesis, was included as a positive control in the first experiment. It reduced pyrimidine pools but also led to an increase in purine levels. Con A stimulated rat spleen cells cultured with 1 to 10 μM leflunomide for eighteen hours also exhibited a reduction of CTP and UTP levels (Table 2). At the higher concentrations purine levels were also reduced.

Discussion

In attempting to elucidate the mechanism of action of leflunomide we have focussed on the anti-proliferative activity. Here we report that leflunomide is anti-proliferative towards transformed lymphoid and non-lymphoid cell lines and towards lines derived from several species. Leflunomide also showed anti-proliferative activity towards normal mouse, rat and human lymphoid cell populations stimulated by lymphocyte mitogens. These results are consistent with previous studies [1, 13, 14, 23]. More importantly, we showed that 1) the anti-proliferative activity of leflunomide in all of these populations can be blocked by the addition of exogenous

pyrimidine nucleosides to the culture media when the cultures are initiated and 2) leflunomide decreases pyrimidine nucleotide pools. We used brequinar, another immunosuppressive compound which blocks the fourth enzyme in pyrimidine biosynthesis, dihydroorotate dehydrogenase [24], as a comparison standard in the proliferation and nucleotide pool assays. As with leflunomide, the anti-proliferative effects of brequinar could be completely overcome by uridine, and brequinar reduced pyrimidine pools. Purine nucleosides, which reverse the anti-proliferative activity of the purine nucleotide inhibitor mycophenolic acid [25], had no effect on leflunomide or brequinar activity indicating that neither of these compounds inhibit inosine monophosphate dehydrogenase. The fetal bovine serum in our cultures most likely contains pyrimidine nucleotides although we did not quantitate these levels. If these levels are sufficiently high then the IC_{50} values of leflunomide for proliferation might be artefactually high. Similarly, the degree of reduction in pyrimidine nucleotides may be underestimated in these experiments. However, even with this caveat in mind, these results show that, like brequinar, leflunomide interferes with pyrimidine nucleotide metabolism. The results do not define the enzyme which is affected by leflunomide.

Our data also indicate that a concentration of leflunomide (10 μM) that reduces UTP and CTP pools by 50% after 18 hours only blocks cellular proliferation by 12% at the same time point. Even after 68 hours the proliferation is only reduced by 13%. Thus, other aspects of pyrimidine, and possibly purine, ribonucleotide and deoxyribonucleotide flux besides UTP and CTP pool levels that impinge upon the cascade of events culminating in cellular proliferation may be affected by leflunomide.

The anti-proliferative activity of high concentrations of leflunomide on mouse lymphocytes, the A20-1.11 cells and Jurkat cells was only partially blocked or unaffected by uridine. This was likely due to high dose toxicity in the mouse cells but in Jurkat this activity is only apparent at relatively high but non-toxic concentrations. These observations are consistent with the possibility that leflunomide has additional activities besides the inhibition of pyrimidine biosynthesis. One possibility is the ability of leflunomide to block tyrosine kinase activity [16].

Initially we reasoned that the mechanism of action of leflunomide might be more apparent in freshly isolated rat spleen lymphocytes since they were dramatically more sensitive than normal mouse or human lymphocytes. However, the higher sensitivity is not seen in all rat cells since the rat pheochromocytoma cell line ACH2 is only as sensitive as human or mouse cells. We speculate, but have not proven, that the normal rat lymphocytes are more sensitive to leflunomide because they may have lower cytidine deaminase activity as compared to the other species. Cytidine deaminase can convert endogenous cytidine to uridine which enters into the pathway to form uridine monophosphate. This possibility is supported by the observation that exogenously supplied uridine, but not cytidine, rescues rat lymphocytes from leflunomide's anti-proliferative activity. However, there are six enzymes in the de novo pathway of pyrimidine biosynthesis. Any one or more of these could be susceptible to the action of leflunomide in the rat.

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