The Immunosuppressive Metabolite of Leflunomide Is a Potent Inhibitor of Human Dihydroorotate Dehydrogenase

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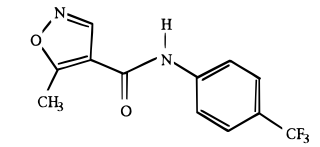
ABSTRACT: The active metabolite of leflunomide, A771726, is a novel immunosuppressive compound that has been shown to be a powerful antiproliferative agent for mononuclear and T-cells. The molecular mechanism of action for this compound has not been clearly established. *In vitro* cellular and enzymatic assays, however, demonstrate that leflunomide is an inhibitor of several protein tyrosine kinases, with IC_{50} values between 30 and 100 μM. The *in vivo* properties of A771726 are reminiscent of another immunosuppressive agent, brequinar sodium, which has been shown to be a nanomolar inhibitor (K_i = 10−30 nM) of the enzyme dihydroorotate dehydrogenase (DHODase). On this basis, we have investigated the effects of leflunomide and A771726 on the activity of purified recombinant human DHODase. We find that A771726 is a potent inhibitor of DHODase (K_i = 179 ± 19 nM), while the parent compound, leflunomide, had no inhibitory effect at concentrations as high as 1 μM. Studies of the dependence of inhibition on the concentrations of the substrates ubiquinone and dihydroorotate demonstrate that A771726 is a competitive inhibitor of the ubiquinone binding site and is noncompetitive with respect to dihydroorotate. The potency of A771726 as a DHODase inhibitor is thus 100−1000-fold greater than that reported for its inhibition of protein tyrosine kinases. These data suggest that an alternative explanation for the immunosuppressive efficacy of A771726 may be the potent inhibition of DHODase by this compound.

Organ transplantation has become an important modern treatment for patients with advanced organ damage. In the United States alone over 100 000 people have received kidney transplants, with patient survival rates of ca. 80−85% after 5 years. This life-sustaining therapy has been successful in large part because of the availability of immunosuppressive drugs that attenuate immune response in the patient, thus overcoming the normal immune-based rejection that would otherwise occur. The two immunosuppressive drugs most commonly used in organ transplantation, cyclosporin A and FK506 (Tacrolimus), share a similar mechanism of action along with some common side effects (Thomson, 1990). There is considerable interest in identifying novel immunosuppressive compounds, with alternative mechanisms of action, to potentially circumvent some of the negative effects of current therapies. Such immunosuppressive compounds could also be useful in the treatment of other immune-based diseases, such as rheumatoid arthritis and autoimmune diseases.

Leflunomide (Figure 1) is a novel immunosuppressive that has demonstrated efficacy in human clinical trials for rheumatoid arthritis (Bartlett et al., 1991). The drug also shows efficacy in animal models of arthritis and autoimmune disease and is effective in blocking rejection after allograft and xenograft transplantation in animals (Bartlett et al., 1991; Williams et al., 1994; Xiao et al., 1994). Studies have demonstrated that leflunomide is actually a prodrug that is processed *in vivo* to the active metabolite A771726 (Bartlett et al., 1991). In cellular assays, A771726 has been shown to inhibit proliferation of mononuclear and T-cells. The immunosuppressive effects of this compound are likely to be a direct result of its ability to block proliferation of immune cells. The specific molecular mechanism of action of A771726 as an immunosuppressive, however, has not yet been elucidated.



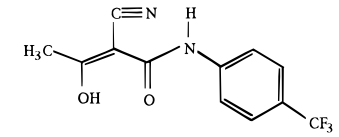


FIGURE 1: Chemical structures of leflunomide and its active metabolite A771726.

Several studies have been reported demonstrating that A771726 is capable of inhibiting a number of protein tyrosine kinases. The IC_{50} values for the compound *in vitro* range from ca. 30 to 100 μM for the various kinases (Bartlett et al., 1991; Mattar et al., 1993; Xu et al., 1995). These data have lead to the widely held view that protein tyrosine kinase inhibition is the molecular mechanism of A771726 immunosuppression *in vivo*. The data that have been reported to date, however, are not sufficient to support this hypothesis unequivocally. In fact, Cherwinski et al. (1995) were not

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able to demonstrate inhibition of protein tyrosine kinases by leflunomide at concentrations that were antiproliferative in T-cells, thus calling into question whether protein tyrosine kinase inhibition is truly relevant to the antiproliferative effects of this compound. These same investigators demonstrated also that the antiproliferative effects of leflunomide could not be reversed by purine nucleosides, a result that distinguished leflunomide from other immunosuppressive agents, such as mycophenolic acid (Cherwinski et al., 1995). In contrast, Nair et al. (1995) recently reported that the antiproliferative effects of leflunomide can be relieved by treatment with uridine in mouse smooth muscle cells. These results imply that the mechanism of antiproliferation by leflunomide involves inhibition of pyrimidine production or use in these cells.

Brequinar sodium (BQR) is another experimental immunosuppressive that also works by blocking immune cell proliferation (Jaffee et al., 1993; Makowka et al., 1993). This compound has been shown (Chen et al., 1992) to function as a nanomolar inhibitor of the enzyme dihydroorotate dehydrogenase (DHODase; L-5,6-dihydroorotate:ubiquinone oxidoreductase; EC 1.3.3.1). By inhibiting this enzyme, brequinar sodium blocks the rate-limiting step in *de novo* pyrimidine biosynthesis. The associated depletion of cellular stores of pyrimidine results in the antiproliferative effects displayed by BQR in cellular assays. As with A771726, brequinar sodium is effective in blocking organ rejection after allograft and xenograft transplantation in animals and is likewise effective in animal models of arthritis and autoimmune disease (Makowka et al., 1993.)

Because of the similarities in the *in vivo* and cellular profiles of A771726 and brequinar sodium, we wondered if the former compound might also function by inhibition of DHODase. We have therefore tested the ability of leflunomide and A771726 to inhibit purified recombinant human DHODase and report the results of these studies here. We find that A771726 is a competititve inhibitor of the ubiquinone binding site of human DHODase, while the parent compound, leflunomide, had no effect on the enzyme. The potency of A771726 as a DHODase inhibitor is much greater than its corresponding potency as a protein tyrosine kinase inhibitor, suggesting that inhibition of DHODase may be an alternative explanation for the immunosuppressive activity of this compound.

MATERIALS AND METHODS

Materials. Recombinant human DHODase was purified as described previously (Copeland et al., 1995). Leflunomide and A771726 were synthesized by the Medicinal Chemistry Department of DuPont Merck Research Laboratories. The identities of the two compounds were confirmed by ^{1}H-NMR, IR, and mass spectroscopy. The purity of each compound was assessed by reverse-phase HPLC and elemental analysis and was found to be > 95% for both compounds. Dihydroorotate (DHO), ubiquinone (coenzyme Q_6; ubiquinone-30), 2,6-dichlorophenolindophenol (DCIP), Triton X-100, gelatin, and Tris buffer were purchased from Sigma.

Enzyme Activity Measurements. DHODase activity was measured by the DCIP colorimetric assay, as described by Copeland et al. (1995). This is a coupled assay in which oxidation of DHO and subsequent reduction of ubiquinone are stoichiometrically equivalent to the reduction of DCIP. Reduction of DCIP is accompanied by a loss of absorbance at 610 nm ($\epsilon = 21\,500\ M^{-1}\ cm^{-1}$). The assay was performed in a 96-well microtiter plate at ambient temperature (ca. 25 °C). Stock solutions of 10 mM leflunomide and A771726 were prepared in dimethyl sulfoxide (DMSO) and these were diluted with reaction buffer (100 mM Tris and 0.1 % Triton X-100, pH 8.0) to prepare working stocks of the inhibitors at varying concentrations. For each reaction, the well contained 10 nM DHODase, 68 μM DCIP, 0.16 mg/mL gelatin, the stated concentration of ubiquinone, 10 μL of an inhibitor working stock to give the stated final concentration, and reaction buffer. After a 5-min equilibration period, the reaction was initiated by addition of DHO to the stated final concentrations. The total volume of reaction mixture for each assay was 150 μL, and the final DMSO concentration was $\leq$ 0.01% (v/v). The reaction progress was followed by recording the loss of absorbance at 610 nm over a 10-min period (during which the velocity remained linear). Velocities are reported as the change in absorbance at 610 nm per minute (in units of mOD/min = $1000\Delta A$/min), and each reported value is the average of three replicates. In experiments where the DHO or ubiquinone concentration was varied, the other substrate was held constant at 200 μM. To determine the inhibitor potency of leflunomide and A771726, the effects of varying concentrations of the two compounds on the initial velocity of the DHODase reaction was measured over a concentration range of 0.01−1.0 μM. In these experiments the DHO and ubiquinone concentrations were held constant at 200 and 100 μM, respectively.

RESULTS AND DISCUSSION

When A771726 is added to a sample of recombinant human DHODase, one observes a concentration-dependent inhibition of enzyme activity with an apparent IC_{50} value of 0.3 μM. Leflunomide, in contrast, did not show any concentration-dependent inhibition of DHODase over the concentration range 0−1.0 μM (data not shown). These data indicated that A771726 is indeed a potent inhibitor of human DHODase. To determine the mode of inhibition of DHODase by A771726, we investigated the effects of substrate concentration on the inhibitory potency of this compound. DHODase requires two substrates for catalysis: dihydroorotate, which is converted to orotic acid by the enzyme, and ubiquinone, which acts as an electron acceptor during catalysis. We therefore varied the concentration of each of these substrates independently while holding the other substrate at a saturating concentration. The initial velocity of the enzymatic reaction at these varying substrate concentrations was determined in the absence of inhibitor and at three different concentrations of A771726: 0.3, 0.6, and 1.0 μM. The data from these experiments were well described by the Henri−Michaelis−Menten equation for each inhibitor concentration (data not shown):

$$v = \frac{V_{max}^{app}[S]}{K_m^{app} + [S]} \quad (1)$$

where K_m^{app} and V_{max}^{app} are the *apparent* values of the kinetic constants at each inhibitor concentration. The data were thus fit to eq 1 by nonlinear least-squares methods to obtain estimates of the kinetic parameters K_m^{app} and V_{max}^{app} for each data set. The values of K_m^{app} and V_{max}^{app} thus derived were

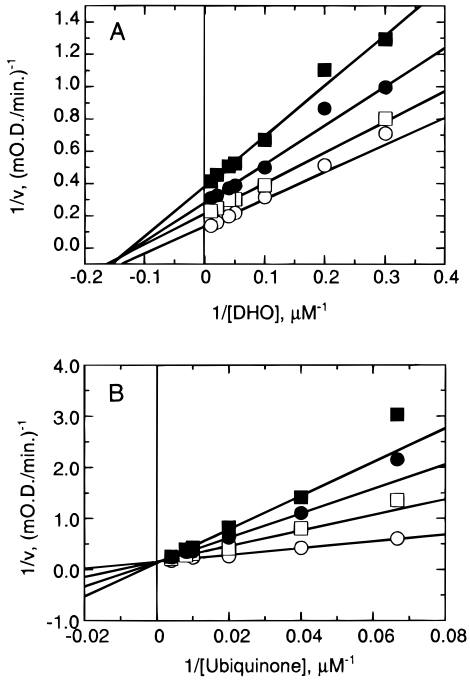

FIGURE 2: Double-reciprocal plots of the initial velocity of DHODase as a function of substrate concentration at varying fixed concentrations of A771726. Panel A illustrates the effects of varying dihydroorotate (DHO) concentration on the initial velocity for four concentrations of inhibitor: no inhibitor (open circles); 0.3 μM (open squares); 0.6 μM (closed circles); and 1.0 μM (closed squares). The lines drawn through the data represent the linear transformation of the K_m^{app} and V_{max}^{app} values from the fit of the untransformed data to eq 1. Panel B illustrates the effects of varying ubiquinone concentrations on the initial velocity for the same four concentrations of inhibitor as in panel A.

used to construct the lines shown in the double-reciprocal plots of Figure 2, according to

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

The patterns observed in panels A and B of Figure 2 are the classical signatures for noncompetitive and competitive inhibition, respectively. From these results we conclude that A771726 is a competitive inhibitor of DHODase with respect to ubiquinone binding and noncompetitive with respect to DHO binding. Knowing that the inhibitor is competitive for ubiquinone binding, we can use the data from Figure 2B to determine the inhibitor constant, K_i, for A771726 from the negative value of the *x*-intercept of a plot of K_m^{app} as a function of inhibitor concentration (Segel, 1975). Figure 3 illustrates such a plot for the data obtained at varying ubiquinone concentrations from Figure 2B. The K_i value determined from this plot was 0.179 ± 0.019 μM.

The K_i values for A771726 inhibition of protein tyrosine kinases have not been reported, making it difficult to correctly compare the relative potency of this compound for DHODase and these enzymes. Comparing the K_i values obtained here for DHODase with the reported IC_{50}s for A771726 inhibition of protein tyrosine kinases, we find that this compound is some 100−1000-fold more potent as a DHODase inhibitor. This comparison is not rigorously valid, however, since the mode of protein tyrosine kinase inhibition has not been reported, and for some inhibitor types (e.g., competitive inhibitors) the substrate concentration can greatly

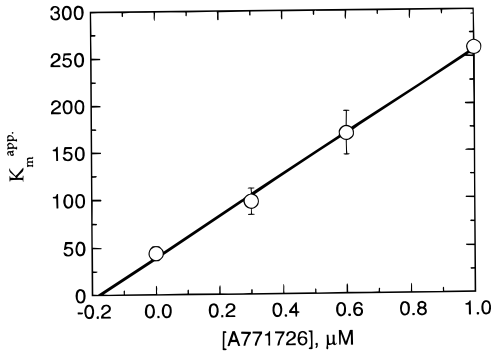

FIGURE 3: Secondary plot of the K_m^{app} for ubiquinone, obtained from the data in Figure 2B, as a function of A771726 concentration. The K_i for the inhibitor was determined from the negative value of the *x*-intercept of the linear least-squares best fit of the data in this figure and was found to be 0.179 ± 0.019 μM.

affect the IC_{50} value obtained (Cheng & Prusoff, 1973). Nevertheless, given the large disparity in these potencies, it is likely that A771726 is a much better DHODase inhibitor than it is a protein tyrosine kinase inhibitor.

While more potent, the DHODase inhibitor BQR demonstrates *in vivo* immunosuppressive efficacy and cellular antiproliferation similar to A771726 (Batt et al., 1995). Both BQR and A771726 block rejection after allograft and xenograft transplantation in animals, both compounds are effective in animal models of arthritis and autoimmune disease, and both compounds inhibit proliferation of peripheral blood mononuclear cells in the mixed lymphocyte reaction assay. Given these similarities in cellular and *in vivo* effects, and the current demonstration that both compounds can potentially block *de novo* pyrimidine biosynthesis by inhibition of DHODase, it seems reasonable to propose that the mechanism of A771726 immunosuppression is based on DHODase inhibition instead of, or in addition to, the ability of this compound to inhibit protein tyrosine kinases.

The mechanism of DHODase inhibition differs between BQR and A771726. BQR has been demonstrated to be a noncompetitive inhibitor of both the DHO and ubiquinone binding sites of the enzyme, with a K_i value of ca. 10−30 nM (Chen et al., 1992; Copeland et al., 1995) Presumably, brequinar sodium inhibits the enzyme by binding at a site that is distinct from both the DHO and ubiquinone binding pockets. In contrast, A771726 is clearly competitive for the ubiquinone binding site of this enzyme (see Figure 2B). Competition for the ubiquinone binding site raises the possibility of poor enzyme selectivity for this compound. Such a poor selectivity profile may lead to broad inhibition of other ubiquinone-dependent enzymes. For example, a general ubiquinone binding inhibitor would be likely to interfere with oxidative phosphorylation by blocking mitochondrial respiration at several key points in the electron transfer cascade. Whether A771726 is a general inhibitor of ubiquinone binding proteins or is specific for the ubiquinone binding site of DHODase remains to be determined. Studies to examine the effects of this compound on mitochondrial electron transfer are currently underway.

In summary, we have demonstrated that A771726 is a potent, competitive inhibitor of human DHODase. The cellular and *in vivo* effects of this compound are all consistent with inhibition of this critical enzyme of the pyrimidine biosynthesis pathway. Whether the *in vivo* immunosuppressive effects of A771726 are due to inhibition of DHODase,

inhibition of protein tyrosine kinases, or the combined effects of inhibiting both enzyme types remains to be established. The present data, however, provide compelling evidence for a potential role of DHODase inhibition in the mechanism of action of this novel immunosuppressive agent.

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