A New Rational Hypothesis for the Pharmacophore of the Active Metabolite of Leflunomide, a Potent Immunosuppressive Drug

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Leflunomide is one of the most promising disease-modifying antirheumatic drug now in clinical trials for the treatment of rheumatoid arthritis. Metabolic studies have indicated that leflunomide is rapidly processed *in vivo* to an active metabolite, A771726 (**2**). To identify the chemical characteristics necessary for the immunosuppressive activity of **2**, configurational and conformational studies were carried out on the latter and its inactive analogues (ethyl 3-hydroxy-2-((4-(trifluoromethyl)phenyl)carbamoyl)but-2-enoate, **3a**, and 3-hydroxy-2-nitro-*N*-(4-(trifluoromethyl)phenyl)but-2-enamide, **3b**). These studies suggested that the pharmacophore responsible for the immunosuppressive activity of **2** is a β-keto amide with the enolic hydroxy group *cis* to the amidic moiety. To verify this hypothesis, a new class of immunosuppressive agents was designed and synthesized. Their testing *in vitro* and *in vivo* identified compounds which were more potent than both leflunomide and **2** and above all confirmed our hypothesis as to the key structural and chemical determinants for the immunosuppressive properties of **2** and our compounds.

Introduction

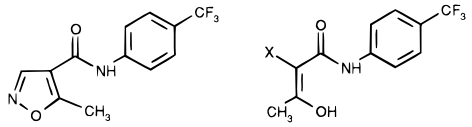
Rheumatoid arthritis (RA), a chronic inflammatory disease of the joints, is a pathology still requiring useful pharmacological approaches. Great effort is directed to the development of the so-called disease-modifying antirheumatic drugs (DMARDs), i.e. drugs which inhibit joint destruction by means of immunomodulating properties. A promising example of DMARDs is represented by leflunomide (HWA 486, **1**, Chart 1), an isoxazole derivative first described more than 15 years ago.[1] Many *in vivo* studies have demonstrated its immunosuppressive and antiinflammatory properties.[2,3] Examples of these activities include the inhibition of (a) adjuvant arthritis in rats,[4,5] (b) systemic lupus erythematosus in MRL/lpr mice,[6,7] (c) experimental allergic encephalomyelitis,[8] (d) glomerulonephritis in rats,[9,10] and (e) chronic graft versus host reaction in mice.[11] Furthermore leflunomide has been found to inhibit transplantation rejection.[2,12]

On the basis of these results, leflunomide has been used in clinical trials and shown to provide clear therapeutic benefit in RA patients.[13]

Metabolic studies have indicated that leflunomide is rapidly processed *in vivo* to the metabolite A771726 (**2**),[2,14] which appears responsible for the activity of leflunomide. Thus both compounds inhibit the proliferation of T cells stimulated by Concanavalin A[15] and the expression of lymphocytes markers which follows cell activation by either phytohemagglutinin (PHA) or an anti-CD3 monoclonal antibody or by the mixed lymphocyte reaction (MLR).[16,17] The mechanism by which **2** exerts its activity has been attributed to the inhibition of tyrosine kinases[18,19] and the subsequent arrest of the cell cycle. As such, **2** should therefore be considered as a cytostatic agent.[2,20]

More recently, however, **2** has been shown to inhibit also the enzyme dihydroorotate dehydrogenase (DHODH),[21,22] a key enzyme in the synthesis of uridine monophosphate. Thus the exact *in vitro* and *in vivo* mechanism of action of this class of compounds remains to be completely defined.[23]

Chart 1



1 Leflunomide

2 X = CN (A771726)

3a X = COOEt

3b X = NO_2

Independently of the precise mechanism of action (tyrosine kinase or DHODH inhibition) the activity of **2**, has been attributed to the presence in its structure of a β-keto nitrile moiety.[23,24] This is because the replacement of the cyano group by either an ethoxycarbonyl[15] (**3a**) or a nitro group[21] (**3b**) has led to inactive derivatives.

While studying the molecular properties of **2** and the inactive derivatives **3a** and **3b**, it was noticed that their low-energy structures differed in the configuration around the enol moiety. On the basis of the hypothesis that this difference could explain the different biological behavior of the compounds and assuming that the immunosuppressive activity of leflunomide is mostly attributable to its metabolite, new derivatives were designed and made. The *in vitro* and *in vivo* results confirmed that the β-keto nitrile group is not essential for the immunosuppressive activity of **2**.

Results and Discussion

Computational Studies. The low-energy structure of **2**, as obtained from a systematic conformational

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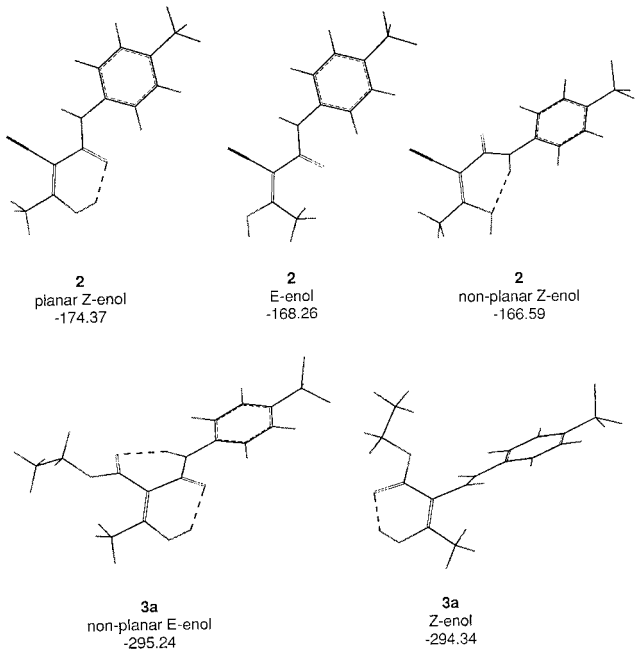


Figure 1. Minimum energy conformations of **2** and **3a** with relative enthalpies of formation (kcal/mol).

search, is characterized by having the enol moiety in a *Z*-configuration. This favors the formation of a six-membered ring stabilized by an H-bond between the hydroxy group of the enol and the C═O of the amide group (Figure 1). This geometry is preferred by about 6 kcal/mol to the *E*-enol, where the above H-bond is no longer possible, and by about 8 kcal/mol to the other *Z*-enol in which, however, the H-bonded six-membered ring is not planar.

When the same calculations were applied to **3a** and **3b**, the *E*-enol configuration, which, due to a different order of substituents, has the same geometry as the *Z*-enol of **2**, was found to be the structure with the lowest energy minimum. As with compound **2**, an H-bond between the hydroxy group and the amidic carbonyl stabilized a six-membered ring. However this ring was distorted, and the lack of planarity was mainly due to the steric hindrance between the methyl group and either the ester or the nitro group. In the case of **3a** an additional H-bonded ring was found on the opposite side of the enolic double bond (Figure 1).

The calculated heat of formation indicated that the *E*-enolic form of **3a** is only less than 1 kcal/mol more stable than the *Z*-enol (Figure 1). The same energy profile was found for **3b**, and in this case the difference between the *E*- and the *Z*-enol was about 2 kcal/mol. These small energy differences suggested that, contrary to **2**, the two enols of both **3a** and **3b** are almost equally populated at room temperature.

These preliminary results seemed therefore to indicate that a structural difference is at the basis of the different immunosuppressive behavior of the molecules. To confirm the role played by the enol configuration with the hydroxy group *cis* to the amidic moiety and a planar six-membered ring, several new compounds were designed and made. In the first such derivative, compound **6**, the possibility of isomerism around the enolic double bond was removed by generating a symmetrical molecule. This removed also the nitrile group of **2** and gave a compound which was more active than the latter

Table 1. Immunosuppressive Activity of Compounds **2**, **3a**, **6**, **8a**−**c**, **11**, and Leflunomide

compound	IC_{50} (μM) mixed lymphocyte reaction
leflunomide	9.6 ± 3.1
6	11.9 ± 0.1
8a	3.4 ± 1.1
8b	1.25 ± 0.2
8c	45.9 ± 29.5
11	1.65 ± 0.1
2	41.3 ± 6.8
3a	71.6 ± 11.3

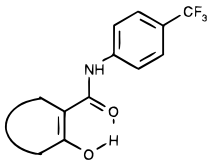


Figure 2. Chemical features important for the immunosuppressive activity of this class of compounds.

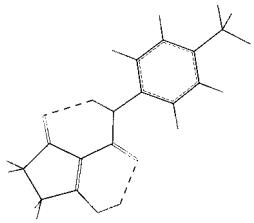


Figure 3. Minimum energy conformation of **8a**.

in an assay measuring their immunosuppressive properties (see below and Table 1). Thus the β-keto nitrile group, the alleged pharmacophore of **2**, did not appear to be necessary for activity.

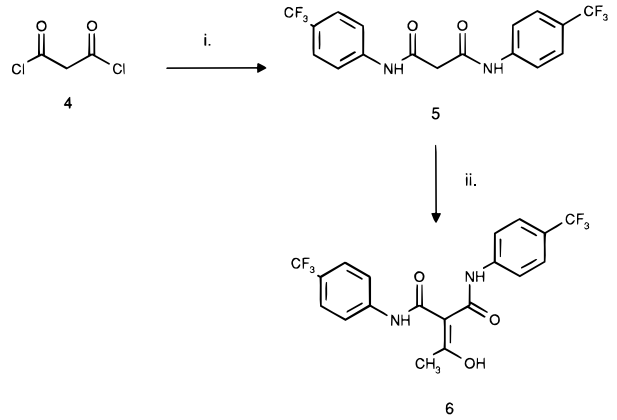
In a second set of compounds the role of the configuration around the double bond was studied. This was done by replacing the β-keto nitrile of **2** with a β-diketone or a β-keto ester moiety appropriately constrained so as to block the enol system into a configuration *cis* to the amide (Figure 2).

As expected, the lowest energy conformers of the new compounds, **8a**−**c**, contained a planar six-membered ring system stabilized by an intramolecular H-bond between the enolic hydroxy group and the amide moiety (Figure 3).

To evaluate whether the structural difference between **3a** and **3b** and the other molecules could be paralleled by their biological behavior and to confirm therefore our structural hypothesis as to the main determinant for the activity of this class of compounds, *in vitro* and *in vivo* studies were carried out.

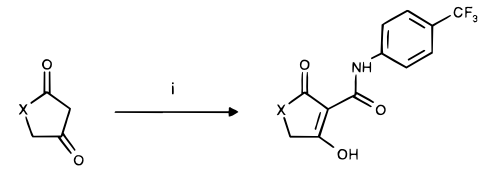
Synthesis. Compound **6** was obtained from malonyl dichloride as outlined in Scheme 1. Briefly, malonyl dichloride was treated with *p*-(trifluoromethyl)aniline, giving malondiamide **5** which was acetylated under basic conditions with sodium hydride and acetylimidazole in tetrahydrofuran at room temperature giving **6**. **8a**−**c** were synthesized in moderate yields, treating the appropriate β-diketone or β-keto lactone, **7a**−**c**, with commercially available 4-(trifluoromethyl)phenyl isocyanate under basic conditions (sodium hydride) in tetrahydrofuran (Scheme 2). **11** was prepared from acetylsalicylic acid by reaction with thionyl chloride first and then with *p*-(trifluoromethyl)aniline, giving amide **10** which was then deprotected to **11** (Scheme 3).

Scheme 1[a]



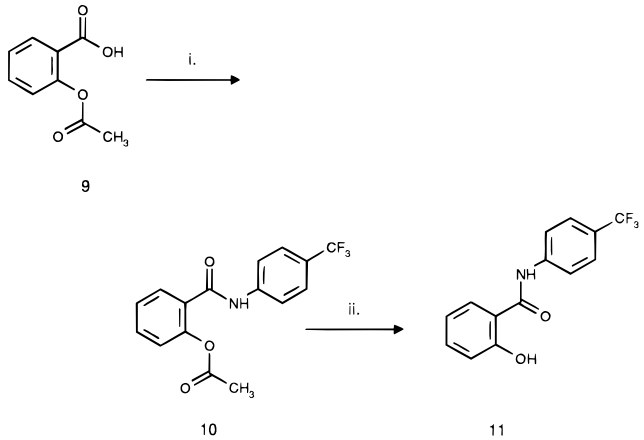
[a] (i) 4-(Trifluoromethyl)aniline, Et_3N, CH_2Cl_2; (ii) NaH, 1-acetylimidazole, THF.

Scheme 2[a]



[a] (i) NaH, 4-(trifluoromethyl)phenyl isocyanate, THF.

Scheme 3[a]



[a] (i) thionyl chloride, reflux, then 4-(trifluoromethyl)aniline, Et_3N, CH_2Cl_2; (ii) imidazole, MeOH.

Biological Activity. The biological activity of these compounds was initially compared to that of leflunomide and **2** in the *in vitro* MLR assay. The latter was chosen as a predictive *in vitro* test for immunosuppressive drugs since it permits us to quantify the reactivity of T cells to an allogenic stimulus. In particular MLR has already been used[17] to (i) characterize the immunosuppressive effects of leflunomide and **2** following reports on efficacy in transplantation models,[2,12] and (ii) study its mode of action on T cell activation and proliferation. The results we obtained for **2** (Table 1) were in agreement with those previously reported.[17]

As anticipated above symmetric compound **6**, which does not possess stereoisomerism around the double bond of the enolic system, was as active (IC_{50} = 11.9 μM) as leflunomide (IC_{50} = 9.6 μM) and more active than **2** (IC_{50}= 41.3 μM). This indicated that the cyano group is not necessary for the immunosuppressive activity and that, in order to exert its activity, the enolic hydroxy group must be *cis* to the amidic moiety.

Table 2. Inhibition of Compounds **2**, **3a**, **6**, **8a**−**c**, **11**, and Leflunomide on CD2, CD25, and CD71 Lymphocyte Activation Antigens Expression

	IC_{50} (μM)		
compound	CD2 expression	CD25 expression	CD71 expression
leflunomide	125.6	130.0	>100
6	15.3	18.4	18.8
8a	13.8	14.8	66.5
8b	2.9	2.9	11.0
8c	95.4	>100	>100
11	3.0	2.4	4.4
2	50.9	89.9	74.6
3a	90.8	96.4	78.1

Cyclic compounds, **8a,b**, were more potent than both **2** and leflunomide, confirming the importance of the *Z*-configuration of the enolic double bound. The small difference between **8a** and **8b** can be explained by the increased percentage of enolization of 1,3-cyclohexanedione compared to that of 1,3-cyclopentanedione.

8c served to further verify the influence of the percentage of enolization on the activity of this class of compounds. Thus it was reasoned that, unlike the two cyclic β-diketones **8a** and **8b**, the β-keto lactone moiety contained in **8c** would reduce enolization of the carbonyl group. As expected, **8c** had a substantially reduced immunosuppressive activity as determined by MLR.

Additional confirmation of the role played by the keto−enolic equilibrium in the activity of **2** was provided by **11**, where the carbonyl group is forced into the enolic form by aromatization of the cycle (Table 1). Thus, **11** was extremely more potent than **2** and leflunomide and together with **8b** was the best representative of this limited series of compounds.

The immunosuppressive properties of these compounds were further assessed by measuring their ability to inhibit the *in vitro* expression of lymphocytes activation antigens induced by a monoclonal antibody against CD3, as a mitogenic stimulus, in the presence of accessory cells.[25] CD25 (Interleukin-2 receptor) and CD71 (transferrin receptor) were studied since it is well-known that activation of T lymphocytes leads to the expression of these two antigens, an event that precedes their clonal expansion.[26,27]

Compound **2** inhibited dose-dependently the expression of CD25 and CD71, confirming its ability to suppress T lymphocytes activation and proliferation.[16,17,19,28]

In addition we also analyzed the expression of CD2, a surface glycoprotein that plays an important role in T cells activation−adhesion processes.[25,29,30]

The results obtained with **2** (Table 2) agreed with those present in literature, with IC_{50} values for CD25 and CD71 expression of about 90 and 75 μM, respectively. Moreover CD2 expression was inhibited with a similar potency (IC_{50} about 50 μM). The results obtained with our compounds were in agreement with those obtained in the MLR assay. Thus **6**, **8a**, **8b**, and **11** were more active than leflunomide on all three parameters tested, with **8b** and **11** the most potent inhibitors of the series (Table 2).

Table 3. Immunosuppressive Activity of **11** and Leflunomide on Antigen-Induced Paw Edema Formation in the Mouse

treatment	scores[a] (mm)	inhibiton of paw edema formation (%)	statistical significance (p)
naive animals	1.94 ± 0.04		
immunized animals	2.87 ± 0.11		<0.001 vs naive
immunized animals (vehicle-treated)[b]	2.72 ± 0.11		<0.001 vs naive
11, 10 mg/kg ip	2.38 ± 0.07	43	0.003 vs immunized (vehicle-treated)
leflunomide, 10 mg/kg po	2.45 ± 0.14	45	0.014 vs immunized
CsA, 5 mg/kg ip	2.42 ± 0.12	48	0.007 vs immunized
CsA, 50 mg/kg ip	2.10 ± 0.06	83	0.002 vs immunized

[a] Thickness of paw. Mean ± standard error. [b] DMSO was used as vehicle for **11**.

These data further confirmed the hypothesis that the pharmacophore of this class of immunosuppressive compounds is a β-keto amide with the enolic system fixed in a configuration *cis* to the amidic moiety (Figure 2).

11, the most active compound *in vitro*, and leflunomide were tested *in vivo* (Table 3) in a murine model of antigen-induced paw edema formation.[31] **11** was administered ip due to its poor bioavailability after oral administration. This compound (10 mg/kg/day) had an immunosuppressive effect comparable to that of identical doses of leflunomide. The immunosuppressive cyclosporin A (CsA), used as positive control at 5 and 50 mg/kg, inhibited paw edema formation by about 50 and 80%, respectively.

Conclusion

Previous studies had highlighted the importance of the β-keto nitrile group in the immunosuppressive activity of **2**, the so-called active metabolite of leflunomide thought to share with the latter most of its biological properties. This conclusion was derived from a structure−activity relationship study where the substitution of the nitrile moiety had led to inactive derivatives.

In this work we have shown that the immunosuppressive activity of **2** is independent of this group. Thus modeling studies indicated that the reported different biological behavior could be accounted for by the ability shown by **2** but not by its inactive derivatives (**3a,b**) to form a structure containing the enolic hydroxy group *cis* to the amide and thus forming a planar ring system stabilized by an intramolecular H-bond. This hypothesis was confirmed by the results obtained with molecules lacking the β-keto nitrile moiety and appropriately constrained so as to maintain the above structure.

It should be emphasized, however, that our hypothesis is not in contrast with the previous one whereby the β-keto nitrile should represent the pharmacophore of **2**. Thus the β-keto nitrile is only a particular case of our hypothesis.

Finally these results could suggest ways to develop new drugs useful in the treatment of immunological disorders such as RA.

Experimental Section

Melting points were determined on a Buchi apparatus in glass capillary tubes and are uncorrected. Thin-layer chromatography was performed on silica gel glass-backed plates (5719) (E. Merck & Co.), and flash chromatography was performed on silica gel 60 (230−400 mesh ASTM) (E. Merck & Co). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer at 200 and 50.3 MHz, respectively, and the chemical shifts are given in ppm (δ) referenced to DMSO-d_6 (2.50 ppm for the ^{1}H NMR spectra and 39.5 ppm for the ^{13}C NMR). Elemental analyses were carried out on a Perkin-Elmer 240 instrument.

***N,N*-Bis-(4-(trifluoromethyl)phenyl)malondiamide (5).** Malonyl dichloride (13.8 mL, 142 mmol) was slowly added at 0 °C to a solution of 4-(trifluoromethyl)aniline (42.6 mL, 340 mmol) and triethylamine (39.4 mL, 284 mmol) in dichloromethane (500 mL). The mixture was stirred overnight at room temperature, poured into ethyl acetate (1000 mL), and washed successively with water (500 mL), 1 N NaOH (500 mL), and 3 N HCl (3 × 500 mL). The organic phase was dried with anhydrous sodium sulfate and evaporated under reduced pressure, giving 53 g (95%) of pure **5** as a yellow solid: mp 215−217 °C dec; ^{1}H NMR (DMSO-d_6) δ 7.84 (d, 4H), 7.71 (d, 4H), 3.60 (s, 2H). Anal. ($C_{17}H_{12}F_6N_2O_2$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-2-((4-(trifluoromethyl)-phenyl)carbamoyl)-3-hydroxybut-2-enamide (6).** A tetrahydrofuran (100 mL) solution of **5** (14g, 36 mmol) was slowly added at 0 °C to a suspension of sodium hydride 80% oil dispersion (2.15 g, 89.5 mmol) in tetrahydrofuran (150 mL). The mixture was stirred at room temperature for 15 min, 1-acetylimidazole (5.9 g, 54 mmol) in tetrahydrofuran (100 mL) was added, and the mixture was stirred overnight at room temperature. The solution was poured into ethyl acetate (500 mL), and the organic phase was washed with 1 N HCl (400 mL), dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The crude product was tritured twice with hot diethyl ether (50 mL) and filtered, thus giving 8.45 g (55%) of pure **6** as a white solid: 170−174 °C dec; ^{1}H NMR (DMSO-d_6) δ 10.68 (s, 2H), 7.82 (m, 4H), 7.72 (m, 4H), 5.02 (s, 1H), 2.31 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 199.7, 164.2, 142.5, 126.5, 124.6, 124.0, 119.3, 68.7, 30.0. Anal. ($C_{19}H_{14}F_6N_2O_3$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-4-hydroxy-2-oxo-5*H*-furan-3-carboxamide (8c).** A solution of 4-hydroxy-2(5*H*)-furanone **7c** (1.47g, 14.7 mmol) in tetrahydrofuran (30 mL) was slowly added at 0 °C to a suspension of sodium hydride 80% oil dispersion (530 mg, 14.7 mmol) in tetrahydrofuran (30 mL). The mixture was stirred at room temperature for 15 min, a solution of 4-(trifluoromethyl)phenyl isocyanate (2.75 g, 14.7 mmol) in tetrahydrofuran (20 mL) was added, and the mixture was stirred overnight at room temperature. At the end of the reaction, the insoluble product was filtered off and the solvent removed under reduced pressure. Crude product was suspended in water (60 mL) and methanol (6 mL) and the mixture acidified to pH 2 with concentrated HCl; the solid thus obtained was filtered and crystallized from acetone, giving 1.75 g (41%) of pure **8c** as a white solid: 204−206 °C dec; ^{1}H NMR (DMSO-d_6) δ 11.50 (s, 1H), 10.23 (s, 1H), 7.83 (m, 2H), 7.65 (m, 2H), 4.71 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 187.6, 173.5, 160.2, 142.5, 126.3, 124..6, 123.3, 119.9, 92.7, 68.1. Anal. ($C_{12}H_8F_3NO_4$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-2-hydroxy-5-oxocyclopentanecarboxamide (8a).** This compound was obtained as a white solid (yield 32%) starting from 1,3-cyclopentanedione and using the same procedure as for compound **8c**: mp 212−214 °C dec; ^{1}H NMR (DMSO-d_6) δ 10.53 (s, 1H), 7.85 (m, 2H), 7.71 (m, 2H), 2.26 (s, 4H); ^{13}C NMR (DMSO-d_6) δ 201.2, 162.5, 141.8, 126.5, 124.6, 123.9, 119.9, 108.2, 30.84. Anal. ($C_{13}H_{10}F_3NO_3$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-2-hydroxy-6-oxocyclohexanecarboxamide (8b).** This compound was obtained as a white solid (yield 38%) starting from 1,3-cyclohexanedione

and using the same procedure as for compound **8c**: mp 125−127 °C dec; ^{1}H NMR (DMSO-d_6) δ 12.78 (s, 1H), 7.81 (m, 2H), 7.75 (m, 2H), 2.64 (m, 4H), 1.95 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 197.0, 169.9, 140.4, 126.6, 125.1, 124.4, 121.4, 103.4, 34.4, 18.9. Anal. ($C_{14}H_{12}F_3NO_3$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-2-acetoxybenzamide (10).** Acetylsalicylic acid (10 g, 55 mmol) was added to thionyl chloride (8.1 mL, 110 mmol), and the suspension was refluxed until complete dissolution of the acid. The yellow solution was then cooled at room temperature, and thionyl chloride was evaporated under reduced pressure. Crude acyl chloride was dissolved in chloroform, and the solvent was removed under reduced pressure to give a reddish oil. This procedure was repeated three times. The oil was dissolved in dichloromethane (100 mL) and the resulting solution slowly added at 0 °C to a solution of 4-(trifluoromethyl)aniline (6.3 mL, 50 mmol) and triethylamine (23 mL, 166 mmol) in dichloromethane (100 mL). The mixture was stirred overnight at room temperature, and then the organic solution was washed successively with 1 N HCl (2 × 200 mL), 1 N NaOH (200 mL), and water (200 mL). The organic phase was dried with anhydrous sodium sulfate and evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel (eluent *n*-hexane−ethyl acetate, 8:2), giving 13 g (80%) of pure **10** as a white solid: mp 210−212 °C; ^{1}H NMR (DMSO-d_6) δ 10.74 (s, 1H), 7.97 (d, 2H), 7.74 (d, 3H), 7.63 (t, 1H), 7.45 (t,1H), 7.30 (d,1H), 2.22 (s,3H). Anal. ($C_{16}H_{12}F_3NO_3$) C, H, F, N.

***N*-(4-(Trifluoromethyl)phenyl)-2-hydroxybenzamide (11).** Imidazole (960 mg, 14 mmol) was added to **10** (13. g, 40 mmol) in methanol (300 mL) and the solution stirred at room temperature for 2 h. The white precipitate was filtered and dried, giving 7.3 g (65%) of pure **11** as a white solid: mp 212−214 °C; ^{1}H NMR (DMSO-d_6) δ 11.51 (s, 1H), 10.67 (s, 1H), 7.98 (m, 2H), 7.93 (dd, 1H), 7.75 (m, 2H), 7.47 (m, 1H), 7.02 (d, 1H), 7.00 (t, 1H); ^{13}C NMR (DMSO-d_6) δ 166.9, 158.1, 142.3, 134.0, 129.6, 126.3, 124.6, 124.2, 120.8, 119.5, 118.5, 117.4. Anal. ($C_{14}H_{10}F_3NO_2$) C, H, F, N.

Configurational and Conformational Studies. 2, **3a,b**, and **8a**−**c** were studied with a systematic search involving all the torsions with 30° steps. Tripos60 force field as implemented in Sybyl6.2 (Tripos) was used without the electrostatic component. The systematic search was set up to filter only the conformers having potential energy within 20 kcal/mol of the minimum. Each conformer was then submitted to geometry optimization through semiempirical calculations (AM1 hamiltonian with keywords: MMOK GNORM=0). Geometrical isomers and/or conformers of each compound with the lower heats of formation were compared. The lowest energy conformers from each compound were then fitted using as reference atoms the six-membered H-bond pattern depicted in Figure 3.

Mixed Lymphocyte Reaction (MLR) Assay. Bidirectional MLR using murine cells was adopted. Splenocytes were prepared from BALB/c and DBA/2 mice (18−20 g) (Charles River, Calco, Italy). Briefly, single-cell suspensions were isolated from spleen by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation; 5×10^4 splenocytes of each preparation were cocultured for 96 h in triplicate in 96-well round-bottom plates (Nunc, Roskilde, Denmark) in the absence or presence of serial dilutions of the tested compounds. All compounds were dissolved in dimethyl sulfoxide (DMSO) to a final 20 mM concentration and then diluted in the culture medium used for the cell assay. To take into account a possible interfering effect of DMSO, each experiment included a control in which the DMSO concentration was made equal to those in compound-treated cultures. Culture medium was RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 5% of fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), 50 μM 2β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate sodium, 100 IU/mL penicillin, and 100 μg/mL streptomycin. During the last 18 h of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, UK) and then harvested onto glass fiber filters by means of a cell harvester. Radioactivity, proportional to proliferation, was measured with a β-counter. Inhibitory activity was expressed as IC_{50}, the concentration of compound that exerted 50% inhibition of proliferation with respect to that of untreated control cultures. All the experiments were repeated at least three times.

Inhibition of Lymphocyte Activation Antigens Expression. Human peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-Hypaque density-gradient centrifugation. The cells recovered were resuspended in culture medium (CM): RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

PBMC, suspended at 10^6 cells/mL in CM, were incubated in 24-well plates (Costar, Cambridge, MA) with the tested compounds and stimulated with optimal concentration of the monoclonal antibody OKT3 (Ortho Diagnostic Inc., Raritan, NJ) for 24 h at 37 °C and 5% CO_2. After incubation, immunofluorescence staining procedure for CD2, CD25, and CD71 antigens and flow cytometry analysis were performed as previously described.[25] The experiments were repeated at least twice, and the difference in IC_{50} values was less than 10%.

Antigen-induced Paw Edema In The Mouse. Groups of four to eight female BALB/c mice (20−22 g; Charles River, Calco, Italy) were immunized by two sc injections on days 0 and 7 of keyhole limpet hemocyanin (KLH, 100 μg/mouse; Sigma Chemicals, St. Louis, MO) emulsified in 0.2 mL complete Freund's adjuvant (CFA, Sigma Chemicals, St. Louis, MO). Nonimmunized mice (Control) were treated with CFA alone. On day 14 immunised mice were challenged in the hind footpad with 20 μg of KLH suspended in physiological solution. Eight hours after antigen challenge the edema was quantified by measuring the dorsal−ventral thickness of the footpad with a scientific micrometer (Borletti, Italy**)**. To evaluate the immunosuppressive activity, the compounds were administered daily from day 0 until day 13. **11**, dissolved in DMSO, was administered ip; leflunomide, suspended in carboxymethylcellulose, was administered po; cyclosporin A was dissolved in physiological solution containing 18% ethyl alcohol and 2% Tween 80 and administered ip. The potencies of the compounds were expressed as percentage of inhibition of edema formation calculated as follows:

$$100 - \frac{(\text{thickness of immunized animals treated with compounds} - \text{thickness of naive animals})}{(\text{thickness of immunized animals} - \text{thickness of naive animals})} \times 100$$

Statistical analysis was carried out by unpaired T-test with Welch correction using GraphPad 2.01 software (Intuitive Software for Science Inc., San Diego, CA).

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