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ISOXAZOLYLTHIOAMIDES AS POTENTIAL IMMUNOSUPPRESSANTS A COMBINATORIAL CHEMISTRY APPROACH

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Abstract: A library of thioamide derivatives of leflunomide **1a** and of its bioactive metabolite **1b** has been synthesised on solid phase. Thus, para-substituted phenylacetic acids were coupled to TentaGel and were subsequently reacted with aromatic isothiocyanates. Treatment of the resulting enaminothioamides with hydroxylamine led to their simultaneous cyclisation and cleavage from the resin affording **2-25**. Their *in vitro* profiling demonstrated that the amide-thioamide isologous substitution was detrimental of the biological activity.

The immunosuppressive activity of leflunomide **1a** is due to its metabolite **1b** which is rapidly formed *in vivo* [1]. In vitro, the effects of **1b** have been shown to be mediated through the inhibition of dihydroorotate dehydrogenase (DHODH) [2] but, at higher concentrations (>50-fold), similar to those obtained *in vivo*, the compound also inhibits a series of tyrosine kinases [3]. In view of the lack of 3D information on human DHODH [4] and of the complex biochemical mechanism of action of leflunomide, we embarked on a program to probe both the DHODH inhibitory properties as well as the immunosuppressive activities of the corresponding thioamides (Figure 1). Indeed, although the introduction of a thioamide group is an isologous substitution for an amide bond [5], several studies have shown that the consequences of this replacement on biological activity is unpredictable [6]. To gain insight into the modulation of the biological activity generated by this modification, the outcome of vinylic and of aromatic substitution was also investigated. For the latter, only para substituents were introduced since ortho substituents would abolish the planarity required for biological activity in the leflunomide series.[7] Also meta substituents have been shown to little influence on the biological activity [8].

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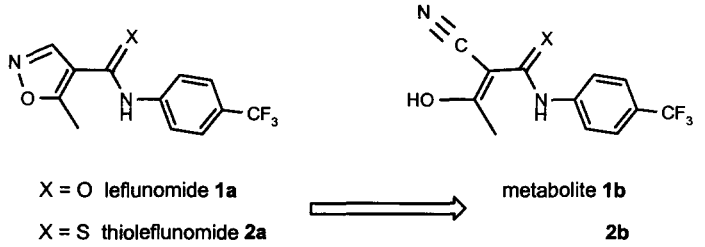
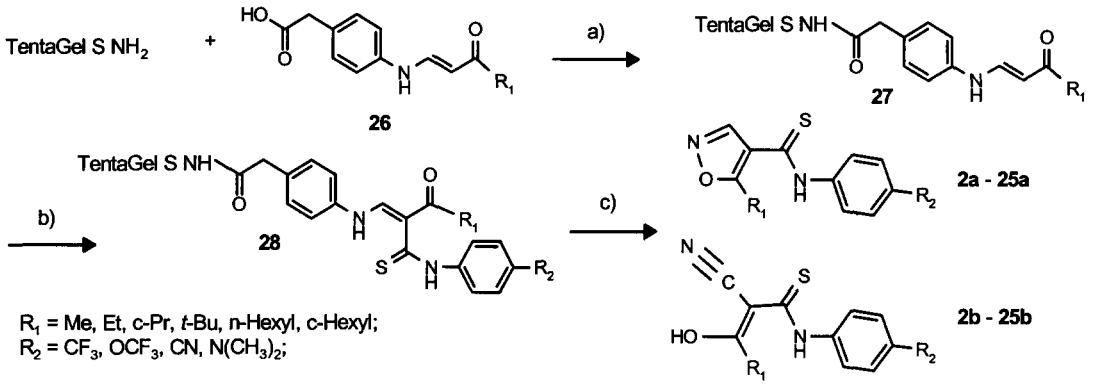
Figure I. Structures of leflunomide **1a**, its metabolite **1b** and of their thioamides analogues **2a** and **2b** respectively.



Consequently, a combinatorial chemistry approach [9] was devised allowing the synthesis of a library of aromatic isoxazolylthioamides **2a - 25a** and of the corresponding 2-cyano-3-hydroxypropen-thioamides **2b - 25b** (Scheme I).

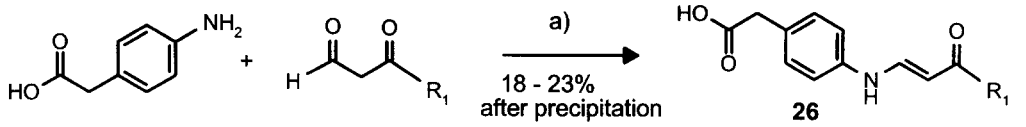
Scheme I. Solid phase synthesis of isoxazolylthioamides and cyanohydroxypropenamides.



a) DCCI/HOBT; b) p-R_2-phenylisothiocyanate (90° C); c) $H_2NOH.HCl/NaHCO_3$, water/ethanol (50° C);

The key issue for the efficient compound production via the above scheme is the availability of para-substituted phenylacetic acid building blocks **26**. The literature procedures allowing their preparation are straightforward and employ commercially available starting materials (Scheme II) [10, 11, 12] . However, they require long reaction times (typically 3 days) and afford **26** in low isolated yields after precipitation of the compounds from the reaction mixture upon addition of diethyl ether.

Scheme II. Solution synthesis of anilinophenylacetic acid building blocks **26**.



a) Na^0, H_3CCOR_1, $HCOOCH_3$, c-hexane R_1 = Me, Et, c-Pr, *t*-Bu, n-Hexyl, c-Hexyl;

Therefore, to avoid an initial low loading of the resin it was decided to prepare **26** in solution and to also proceed with its coupling on the resin (TentaGel S NH_2 resin, RAPP Polymere, S 30902) [13] employing standard methods of solid phase peptide synthesis [14]. The reaction of the immobilised enamine **27** with a 50% THF solution of various p-substituted phenylisothiocyanates under reflux allowed the introduction of the second building block. The cyclisation to the final isoxazolyl thioamides and the simultaneous cleavage from the resin was achieved by treating the resin with hydroxylamine hydrochloride in aqueous ethanol at 50°C (pH 7; $NaHCO_3$). After removal of the solvent, the residue was partitioned between ethylacetate and water. The organic phase was evaporated to dryness and the resulting crude compounds were sufficiently pure for biological evaluation without any further purification. In general 500 mg of resin (loading ~ 0.2-0.3 mmol/g) were used for each reaction sequence in order to guarantee 5 to 10 mg final product (~12-20 % overall yield). According to HPLC analysis, isoxazolethioamides were predominantly obtained (>95%) and their structures were confirmed by FAB-MS. This combinatorial chemistry approach enabled the preparation of a biased library of 25 thiocarboxamide analogues of leflunomide.

For the direct comparison of the biological activities of **1a** and **1b** with their thioamide analogues, pure **2b** was obtained upon hydrolysis of **2a** [15] with 1.25 equivalents of solid NaOH in methanol at 60° C for 90 minutes. The compounds were evaluated for their *in vitro* biological activities in two assays (Table 1). First, their inhibitory activity on human, recombinant DHODH was determined [16] and second their immunosuppressive activity was measured in the mouse mixed lymphocyte reaction (MLR) [17]. The former is a rapid cell free assay and was employed to check the biological similarity of the four compounds while the latter allowed the detection of substances interfering with the proliferative response of lymphocytes.

Table 1. Comparative biological activities [a]

Compound	DHODH [b]	MLR [b]
leflunomide **1a**	10	10
metabolite **1b**	0.2	10
2a	>50	23
2b	0.8	11

[a] Mean of 3 independent experiments. [b] IC_{50} in μM .

Indeed, concerning immunosuppresion **2a** is two times less active than **1a**, whereas **1b** and **2b** is equipotent. Concerning enzyme inhibition, **2a** fails to inhibit DHODH while **2b** is a four time weaker inhibitor than the corresponding amide. In view of the lack of any information about the structure of human DHODH and the mode of binding of leflunomide metabolite to it, no rational explanations for the reduced activity of the thioamides could be proposed. However, the results demonstrate that **2a**, the isoxazolyl thioamide analogue of leflunomide, can inhibit T cell proliferation by a mechanism which is independent of DHODH inhibition. It remains to be verified if this mechanism, which operates at low μM concentrations, is kinase based as is the case for high concentrations of leflunomide [3] .

The data obtained with the crude thioamides **3a** - **25a** (Table 2) [18] demonstrate that the compounds having bulky R_1 moieties (*tert*-butyl, hexyl, cyclohexyl) are inactive and that those with R= $N(CH_3)_2$ show only marginal activity. However, in contrast to the inactive thioleflunomide **2a**, derivatives **7a** and **11a** having the p-trifluoromethoxyaniline group in common are sub-micromolar enzyme inhibitors. Their activity is comparable to that of the cyanopropenthioamide **2b**. Surprisingly, **3a**, the corresponding compound in the methyl series, is approximately 9 times less active indicating that a lipophilic pocket of moderate size must be present in the vicinity of position 5 of the isoxazolyl ring.

The ensemble of the structure-activity relationships described together with the limited data reported on DHODH inhibition with cyanopropenamides [7, 8] emphasises that no predictions concerning the outcome of the amide-thioamide isologous substitution can be made, particularly in the absence of structural information.

Table 2. DHODH inhibitory potency of compounds **2a - 25a** (IC_{50} in μM)

Compound	R_1	R_2	DHODH[a]
2a	Me	$-CF_3$	> 50
3a	Me	$-OCF_3$	7.2
4a	Me	-CN	35
5a	Me	$-N(CH_3)_2$	> 50
6a	Et	$-CF_3$	3
7a	Et	$-OCF_3$	0.7
8a	Et	-CN	11
9a	Et	$-N(CH_3)_2$	40.4
10a	c-Pr	$-CF_3$	3.2
11a	c-Pr	$-OCF_3$	0.8
12a	c-Pr	-CN	9.3
13a	c-Pr	$-N(CH_3)_2$	> 50
14a	*t*-Bu	$-CF_3$	> 50
15a	*t*-Bu	$-OCF_3$	> 50
16a	*t*-Bu	-CN	> 50
17a	*t*-Bu	$-N(CH_3)_2$	> 50
18a	n-Hexyl	$-CF_3$	> 50
19a	n-Hexyl	$-OCF_3$	> 50
20a	n-Hexyl	-CN	> 50
21a	n-Hexyl	$-N(CH_3)_2$	> 50
22a	c-Hexyl	$-CF_3$	> 50
23a	c-Hexyl	$-OCF_3$	> 50
24a	c-Hexyl	-CN	> 50
25a	c-Hexyl	$-N(CH_3)_2$	> 50
1a (leflunomide)			10
1b (metabolite)			0.2

[a] Mean of 3 independent experiments

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15. Physical data for analytically pure compounds:
    ^{1}H-NMR (DMSO-d_6) data for **2a**: d 2.70 (3H, s, CH_3), 7.80 (2H, d, J = 8.2 Hz, aromatic), 8.10 (2H, d, J = 8.2 Hz, aromatic), 8.90 (1H, s, isoxazole), 11.80 (1H, s, NH)
    ^{1}H-NMR (DMSO-d_6) data for **2b**: d 2.10 (3H, s, CH_3), 7.60 (2H, d, J = 8.2 Hz, aromatic), 8.10 (2H, d, J = 8.2 Hz, aromatic), 9.75 (1H, broad, OH)
    HPLC conditions and retention times (R_t) :
    Column : Spherisorb 4.5 x 125 mm ; UV detection λ = 240 nm ; Flow : 1.25 ml/min
    Eluent : H_2O / CH_3CN (+1%TFA) ; Gradient : 100/0 to 20/80 in 25 min.
    R_t for **2a** :19.15 min. R_t for **2b** :14.56 min.
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