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The Synthesis and Selective IL-2 Inhibitory Activity of Bis Piperazine–Phenol Mannich Adducts

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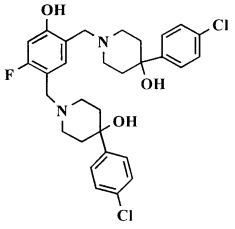
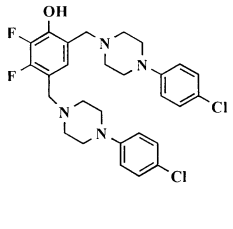
Abstract—Novel phenol bis-Mannich adducts were identified as IL-2 expression inhibitors in a T cell proliferation screening assay. Analogues of the lead compound were prepared through parallel synthesis and a highly selective IL-2 inhibitor was discovered that provided a suitable compound for further optimization.

T cell activation plays a pivotal role in the immune response.[1] Inhibition of the signal transduction or any critical events in this activation cascade would be clearly beneficial for the treatment of organ transplant rejection or autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Systemic treatment with the commercially available therapeutics cyclosporin A and FK506, both of which interfere with the Ca^{++}-sensitive T cell activation signal transduction pathway, results in liver and renal dysfunction among other side effects.[2–4] Despite extensive studies, the underlying mechanism of toxicity of these agents is still not well understood. One hypothesis is that inhibition of the key signaling enzyme calcineurin is responsible for the observed toxicity.[5] Therefore, a small-molecule immunosuppressant working via a mechanism that differs from that of cyclosporin A and FK506 might be free of the current drugs' side effects.[6–8] In a joint drug discovery effort of ArQule and AVANT, ArQule's Mapping Array™ collection was screened in a cell-based assay using T cells transfected with a reporter gene whose expression is regulated by a lymphocyte activation sensitive cytokine enhancer/promoter. A number of relatively potent hits were identified, such as compound **Ia** (Fig. 1). Our discovery goal is not only to retain or increase the potency of IL-2 expression inhibition, but also to discover a compound with much higher selectivity over β-actin. In this communication, we report our initial optimization results achieved using ArQule's Directed Array™ program that resulted in the highly selective compound **IIa**.

Based on the primary screening hit **Ia**, analogues (see Fig. 2) were synthesized to improve the potency and selectivity, while also addressing solubility issues. As shown in Scheme 1, the parallel synthesis of this class of compounds was achieved in modest to good yields by allowing the phenol, formaldehyde and substituted piperidines or piperazines to react under Mannich reaction conditions.[9] The regiochemistry of the Mannich adducts was determined by ^{1}H NMR. The products were purified by HPLC and characterized by LC–MS.[10] In the case of 2-fluoro, 2,5-difluoro, 2,3–difluoro, and



Ia



IIa

Figure 1.

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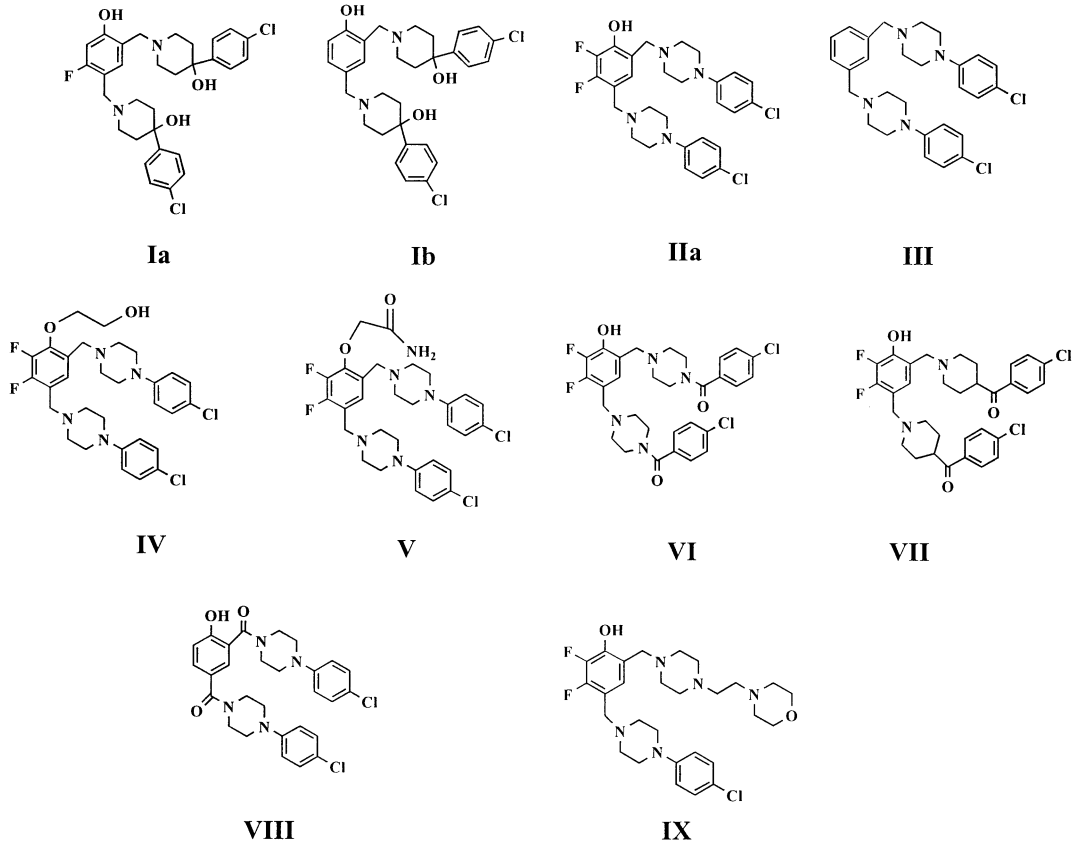
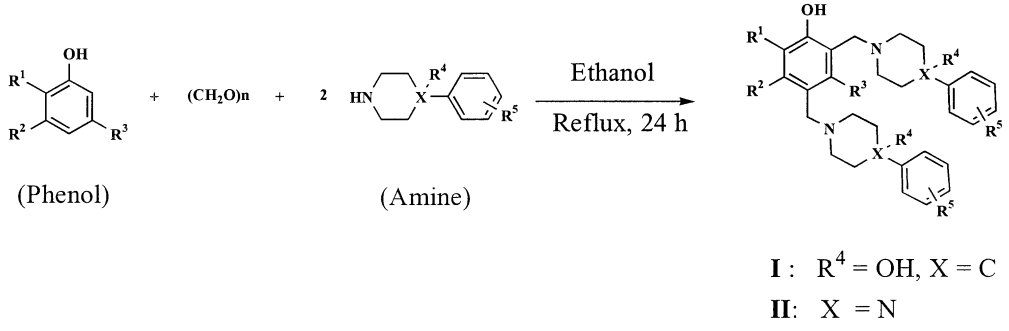


Figure 2.



Scheme 1.

2,3,5-trifluoro-substituted phenols, disubstitution at the 4 and 6 positions is the only possibility. In the case of 3-fluoro and 3,5-difluoro-substituted phenols (**If**, **Ih**, **Ii**, **IIb**, and **IId**) we observed formation of two–three disubstituted isomers that were not readily separated by HPLC. These were tested as the mixture of disubstituted products. Compound **Ia** was isolated from a mixture of two disubstituted isomers and one trisubstituted isomer by preparative HPLC. The di- or trisubstituted isomers were easily distinguished by mass spectroscopy. The two disubstituted isomers were differentiated by ^{1}H NMR. In the case of **Ib**, only the 2,4-disubstitution product was isolated. In order to explore some key SAR features, a number of analogues were synthesized individually. Compound **III** was obtained by reductive amination of isophthaldehyde and 4-(4-chlorophenyl) piperazine. Compounds **IV** and **V** were synthesized by alkylation of **IIa**. Compound **VIII** was readily prepared through an amide coupling of 4-hydroxy isophthalic acid and 4-(4-chlorophenyl) piperazine. We have also synthesized a library of unsymmetrical analogues using two different amines (see compound **IX**) via two sequential Mannich reactions.

The primary screen was a cell-based assay using a human T cell lymphoma cell line (Jurkat) that was stably transfected with a DNA fragment carrying the firefly luciferase reporter gene downstream from the IL-2 promoter/enhancer, which contains transcription factor binding sites for NF-ATp, AP-1, and NFκB. The Jurkat-IL2-Luc cells were activated by plate-bound anti-TCR antibody (16G8) plus PMA in the presence of 5 μM of a test compound. IL-2 inhibitory activity was determined quantitatively by luciferase expression using LucLite (Packard) reagents. Compounds with inhibitory activity were resynthesized and the IC_{50} values

Table 1. IL-2 and β-actin inhibitory activity

Compd	Substitutions R^1, R^2, R^3	Amines	IL-2, IC_{50} (μM)[a]	β-Actin, IC_{50} (μM)[a]	Selectivity[b]
Ia	$R^1=R^3=H$, $R^2=F$	4-(4-Chlorophenyl)-4-hydroxy piperidine	1.5	10	6.7
Ib	$R^1=R^2=R^3=H$	4-(4-Chlorophenyl)-4-hydroxy piperidine	1.0	4	4
Ic	$R^2=R^3=H$, $R^1=F$	4-(4-Chlorophenyl)-4-hydroxy piperidine	0.6	5	8.3
Id	$R^1=R^2=F$, $R^3=H$	4-(3-Trifluoromethylphenyl)-4-hydroxy piperidine	6.5	>50	>7
Ie	$R^1=R^3=F$, $R^2=H$	4-(4-Chlorophenyl)-4-hydroxy piperidine	4	NT	NT
If	$R^2=R^3=F$, $R^1=H$	4-(4-Chlorophenyl)-4-hydroxy piperidine	0.8	10	12.5
Ig	$R^2=R^3=H$, $R^1=F$	4-(3-Trifluoromethylphenyl)-4-hydroxy piperidine	0.2	2	10
Ih	$R^1=R^3=H$, $R^2=F$	4-(3-Trifluoromethylphenyl)-4-hydroxy piperidine	1.8	2.5	1.4
Ii	$R^1=R^3=H$, $R^2=F$	4-(4-Methylphenyl)-4-hydroxy piperidine	2	10	5
IIa	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorophenyl) piperazine	0.4	>50	>100
IIb	$R^1=R^3=H$, $R^2=F$	4-(4-Chlorophenyl) piperazine	>5	NT	NA
IIc	$R^2=R^3=H$, $R^1=F$	4-(4-Chlorophenyl) piperazine	3	10	3.3
IId	$R^2=R^3=F$, $R^1=H$	4-(4-Chlorophenyl) piperazine	2	3	1.5
IIe	$R^1=R^3=F$, $R^2=H$	4-(4-Chlorophenyl) piperazine	8	>50	>6.3
IIf	$R^1=R^2=R^3=F$	4-(4-Chlorophenyl) piperazine	3.5	>50	14.3
III	$R^1=R^2=R^3=H$	4-(4-Chlorophenyl) piperazine	2	>40	>20
IV	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorophenyl) piperazine	0.8	NT	NA
V	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorophenyl) piperazine	3	NT	NA
VI	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorobenzoyll) piperazine	>5	NT	NA
VII	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorobenzoyll) piperidine	2	NT	NA
VIII	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorophenyl) piperazine	0.9	5	>6
IX	$R^1=R^2=F$, $R^3=H$	4-(4-Chlorophenyl) piperazine and 4-(4-mopholino)ethyl piperazine	80% at 5 μM	NT	NA

[a]IC_{50} values are the average of three experiments.
[b]The selectivity values were calculated by dividing the IC_{50} of the β-actin assay by the IC_{50} of the IL-2 assay.
>, greater than; NT, not tested; NA, not available.

were determined. The resynthesized compounds were also analyzed for the ability to inhibit β-actin promoter-driven luciferase expression in order to distinguish between specific IL-2 inhibitory activity and nonspecific cytotoxic effects. The selectivity of each of the inhibitory compounds was defined as the ratio of the IC_{50} value for β-actin to IL-2 promoter-driven expression (Table 1).

The IL-2 and β-actin inhibitory activities are shown in Table 1. The results show that the piperazine analogue **IIa** is much more selective than all of the 4-hydroxy-piperidine analogues. In both **I** and **II** series, chlorine, trifluoromethyl, and methyl substitution on the terminal phenyl groups increased potency. In series **II**, nitro and acetyl substituents also afforded better potency but not selectivity (over 70% inhibition at 5 μM screening concentration, data not shown in Table 1). The phenolic hydroxyl group is not critical for potency and selectivity; compound **III** lacks a phenolic hydroxyl group and is comparable with most phenol analogues. The fluorine substitution on the phenol ring modulates both potency and selectivity in general. The most selective and potent compound **IIa** was chosen as an initial lead compound for further optimization. Two amide analogues **VI** and **VIII** of the bis-Mannich adduct **IIa** significantly lost IL-2 inhibitory activity. Similarly, compound **VII**, wherein a carbonyl was inserted between the terminal phenyl group and the piperidine ring, also lost potency. In order to improve the solubility of **IIa** under assay condition (aqueous buffer, physiological pH), the phenol in **IIa** was functionalized with solubilizing groups (**IV** and **V**) to little effect. A number of unsymmetrical analogues were prepared (e.g., **IX**), and some showed improved solubility under the assay conditions. Unfortunately, the unsymmetrical analogues did not exhibit sufficient selectivity (data not shown). However, some consistent SAR trends emerged in the unsymmetrical series, such as that electron withdrawing group substitutions on the terminal phenyl ring afford better potency. It should be noted that reliance on a cell-based assay during the course of this research made SAR trends somewhat difficult to interpret. Detailed mechanism of action studies would help to better understand the role this class of inhibitors plays in the inhibitory process.

In summary, a novel T cell proliferation inhibitor has been identified through screening. Analogues of the bis-Mannich lead compound **Ia** were prepared by parallel organic synthesis, resulting in selective and potent IL-2 mediated T cell inhibitors.

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10. **Compound IIa**: white solid ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (d, 2H, $J=7.8$ Hz), 7.25 (d, 2H, $J=7.8$ Hz), 6.82 (d, 2H, $J=4.8$ Hz), 6.86 (d, 2H, $J=4.8$ Hz), 6.77 (dd, 1H, $J=7.5$, $J=2.4$ Hz), 3.80 (s, 2H), 3.55 (s, 2H), 3.20 (m, 8H), 2.65 (m, 8H). MS (M + 1) 547.2.