Phosphodiesterase Type IV Inhibition. Structure–Activity Relationships of 1,3-Disubstituted Pyrrolidines

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Received November 29, 1994®

The synthesis of 1,3-disubstituted pyrrolidines **2** and their activities as type IV phosphodiesterase (PDE) inhibitors are described. Various groups were appended to the nitrogen of the pyrrolidine nucleus to enable structure–activity relationships to be assessed. Groups which render the pyrrolidine nitrogen of **2** nonbasic yielded potent PDE-IV inhibitors. Analogs of amides, carbamates, and ureas of **2** were synthesized to determine the effects that substitution on these functional groups had on PDE-IV inhibitor potency. The structural requirements for PDE-IV inhibitor potency differed among the three classes. A representative amide, carbamate, and urea (**2c,d,h**) were shown to be >50-fold selective for inhibiting PDE-IV versus representative PDEs from families I–III and V. Furthermore, these same three inhibitors demonstrated potent functional activity (IC_{50} < 1 μM) by inhibiting tumor necrosis factor-α (TNF-α) release from lipopolysaccharide (LPS)-activated purified human peripheral blood monocytes and mouse peritoneal macrophages. These compounds were also tested orally in LPS-injected mice and demonstrated dose-dependent inhibition of serum TNF-α levels.

Cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) are ubiquitous cellular second messengers that mediate the biologic responses of cells to a wide range of extracellular stimuli. The actions of these agents are terminated by the superfamily of enzymes termed cyclic nucleotide phosphodiesterases (PDE) that hydrolyze cAMP and cGMP to their corresponding 5′-monophosphates, 5′-AMP and 5′-GMP, respectively. Human PDEs consist of at least five different families (isotypes), and in some cases there are several enzymes (isoforms) within an isotype.[1] PDE isotypes differ in their tissue distribution and regulation. Therefore, selective inhibition of PDE isotypes or isoforms should selectively increase levels of cAMP or cGMP in a nonrandom, tissue selective fashion.[2]

Numerous studies have demonstrated that elevated levels of cAMP in human lymphoid and myeloid lineage cells result in suppression of cell activation.[3] Recently, it has been shown that of the five families of PDE, the type IV PDE (PDE-IV) is the predominant PDE isotype in these cells. PDE-IV is characterized as a cAMP-specific, calcium- and cGMP-insensitive enzyme that is selectively inhibited by rolipram (**1**) ((*R,S*)-4-(3-cyclopentoxy-4-methoxyphenyl)-2-pyrrolidinone).[4] Inhibiting PDE-IV with rolipram can cause an elevation of cAMP in these proinflammatory cells and suppress their activation.[5] For example, it is known that in vitro rolipram inhibits the production and/or release of proinflammatory cytokines in activated human monocytes,[6] prevents activation and proliferation of human T-lymphocytes,[7] and inhibits human polymorphonuclear leukocyte[8] and guinea pig eosinophil[9] respiratory burst. Since sites of inflammation, such as those that occur in asthma and rheumatoid arthritis, are characterized by the presence and activation of multiple types of inflammatory cells, a potent selective PDE-IV inhibitor could be useful for treating chronic and acute inflammatory diseases.[5]

Although rolipram is the prototype selective PDE-IV inhibitor, its potency is not ideal. Thus, we and several other groups have used rolipram as a scaffold to generate related, highly potent PDE-IV inhibitors.[10,11] In this report we describe results demonstrating that PDE-IV inhibitors with severalfold increased potency relative to rolipram can be obtained by reducing rolipram to the 3-substituted pyrrolidine **2a** (R = H) followed by N-acylation to give pyrrolidines of general structure **2**.

Chemistry

Synthesis of pyrrolidines **2** from rolipram was uneventful utilizing **2a** as the pivotal intermediate. Reduction of rolipram using $LiAlH_4$ in tetrahydrofuran (THF) at reflux for 2 h yielded **2a** as an oil in 76% yield. Refluxing the secondary amine with ethyl formate gave **2b** (R = CHO) in 67% yield. All of the amides (**2**, R = COR′) were synthesized by reacting **2a** with the requisite acid chlorides or anhydrides and triethylamine (TEA) in either acetonitrile or dichloromethane (CH_2Cl_2) at 0–23 °C. The carbamates (**2**, R = CO_2alkyl, phenyl), except for the *tert*-butyl carbamate, were prepared by reacting pyrrolidine **2a** with the appropriate chloroformate and TEA in CH_2Cl_2. The *tert*-butyl carbamate **2p** (R = CO_2tBu) was synthesized by treating **2a** with di-*tert*-butyl dicarbonate in CH_2Cl_2 at 23 °C. The disubstituted urea **2h** (R = $CONH_2$) and thiourea **2i** (R = $CSNH_2$) were made by reacting pyrrolidine **2a** with trimethylsilyl isocyanate or trimethylsilyl isothiocyanate, respectively, and 4-(dimethylamino)pyridine in CH_2Cl_2 at 23 °C. The trisubstituted ureas (**2**, R =

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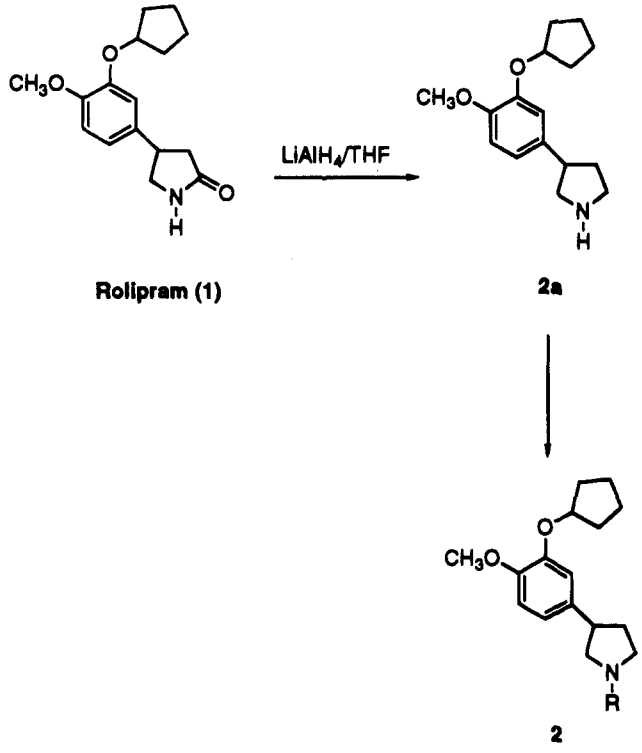
® Abstract published in *Advance ACS Abstracts*, April 1, 1995.

0022-2623/95/1838-1505$09.00/0

Scheme 1. Synthesis of Pyrrolidines **2** from Rolipram



CONHR') were obtained by reacting **2a** with the appropriate isocyanate in acetonitrile or CH_2Cl_2 at 23 °C. The tetrasubstituted urea **2w** (R = $CONMe_2$) was prepared in 49% yield by reacting **2a** with *N,N*-dimethylcarbamoyl chloride and TEA in CH_2Cl_2 at 0 °C. Pyrrolidine **2a** reacted with methanesulfonyl chloride and TEA in CH_2Cl_2 at 0 °C to give the sulfonamide **2e** (R = SO_2CH_3) in 34% yield. The highly enantioenriched carbamates **2ee,ff**, and ureas **2gg,hh** were obtained from (*R*)- and (*S*)-rolipram using the chemistry outlined above for the racemic series.[12,13] Table 1 contains all of the compounds of general structure **2** synthesized, along with some of their physical and biological data.

Biology

It has recently been disclosed that there are four genes encoding type IV PDEs in humans.[14] Furthermore, the diversity of the gene products encoded by the four human loci is augmented by alternative mRNA splicing. In this study, all of the compounds in Table 1 were tested for their ability to inhibit cAMP hydrolysis of the human PDE-IV protein termed PDE type IVb (PDE type IVb is the human homolog of the rat PDE-IV termed dpde4). This protein was recently cloned from a human frontal cortex cDNA library, expressed in the yeast *Saccharomyces cerevisiae*, and purified to functional purity.[15] Selected compounds from Table 1, **2c,d,h**, were tested for inhibition of enzymes from other PDE families. These proteins were purified to functional purity from canine lung[5a] (Table 2). Functional purity indicates that no other PDE activity could be detected in the preparation following purification. All three of the pyrrolidines were tested against PDE-I (± Ca^{2+}/calmodulin), PDE-II (± cGMP), PDE-III, and PDE-V. In order to assess the functional effects of these representative PDE-IV inhibitors, they were tested for their ability to inhibit the production of tumor necrosis factor-α (TNF-α) from lipopolysaccharide (LPS)-stimulated human peripheral blood monocytes[6] (HPBM) and mouse peritoneal macrophages. Furthermore, all three of these compounds were tested orally in LPS-injected mice to determine whether they reduced serum TNF-α levels relative to sham-treated animals (murine serum TNF-α assay). The functional data are tabulated in Table 3. See the Experimental Section for details on how the assays were conducted.

Results and Discussion

The primary structure–activity relationships (SAR) obtained for the compounds synthesized was potency of inhibition of PDE type IVb cAMP hydrolysis by the test compounds. Our goal was to discover compounds with significantly increased potency relative to rolipram in the enzyme, cellular, and murine serum TNF-α assays.

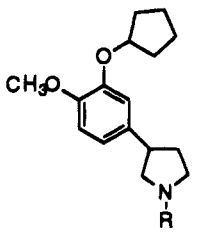
Early in our studies we found that acetylation of rolipram to give **4** generated a PDE-IV inhibitor with potency comparable to that of rolipram ($pK_i = 6.65 \pm 0.24$, $n = 9$ for rolipram; $pK_i = 6.64 \pm 0.10$, $n = 3$ for **4**). Surprisingly, removal of the pyrrolidinone carbonyl from **4** to give **2c** ($pK_i = 7.05 \pm 0.09$, $n = 3$) demonstrated that the pyrrolidinone carbonyl was unnecessary and actually decreased the potency of this class of inhibitors (Figure 1). Therefore, we synthesized various pyrrolidines of general structure **2** to determine what effect changing the functional group R would have on the ability of these compounds to inhibit human PDE-IV. See Table 1.

We surveyed a number of different functional groups (entries 1–10) appended to the pyrrolidine nitrogen of **2**, and all of the pyrrolidines, except for **2g**, demonstrated equivalent or increased potency relative to rolipram. The poor activity of the secondary amine **2l** (oxalate salt of **2a**) suggests that the pyrrolidine nitrogen must be substituted with a group that renders the nitrogen nonbasic in order to be a potent PDE-IV inhibitor.

On the basis of these data, we explored the effects that substituted carbamates, ureas, and amides have on PDE-IV inhibition. As evident from Table 1 (entries 12–17), the ability of the carbamates to inhibit PDE-IV generally increases with increased lipophilicity. Indeed, the *tert*-butyl carbamate **2p** and the phenyl carbamate **2q** are the two most potent PDE-IV inhibitors of all of the compounds tested in this class. Unlike the carbamates, increased lipophilicity on the trisubstituted ureas (entries 18–21) did not increase the PDE-IV inhibitor potency relative to disubstituted urea **2h**. Interestingly, unlike trisubstituted urea **2s**, tetrasubstituted urea **2w** is approximately 2-fold more potent than rolipram and equipotent to the disubstituted urea **2h**. The amides (entries 23–29) show a trend similar to that of the ureas. Increased aliphatic lipophilicity decreases PDE-IV potency relative to the simplest amide, formamide **2b**; however, in general, the aryl amides were equal or slightly more potent than the formamide (entries 25–29).

The enantiomers of methyl carbamate **2d** and urea **2h** were synthesized to determine the effect absolute stereochemistry has on PDE-IV inhibition.[13] The (*S*)-carbamate **2ff** tested as the more potent antipode, although the stereoselectivity is not outstanding. Interestingly, the (*R*)-urea **2gg** is more potent that the

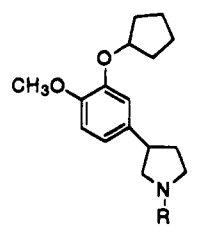
Table 1. Physical and PDE Inhibitory Data on Various Substituted 1,3-Disubstituted Pyrrolidine **2**



entry	comp no.	R[a]	formula[b]	mp (°C)	pK_i[c] ± SD[d] (n)[e]
1	**2b**	CHO	$C_{17}H_{23}NO_3$	oil	7.04 ± 0.06 (4)
2	**2c**	COMe	$C_{18}H_{25}NO_3$	58–60	7.05 ± 0.09
3	**2d**	CO_2Me	$C_{18}H_{25}NO_4$	98–99	6.96 ± 0.18 (4)
4	**2e**	SO_2Me	$C_{17}H_{25}NO_4S$	oil	6.55 ± 0.11 (4)
4	**2f**	COC_2Me	$C_{19}H_{25}NO_5$	78–79	6.71 ± 0.08
6	**2g**	$COCH_2CO_2Me$	$C_{20}H_{27}NO_5$	oil	6.23 ± 0.07
7	**2h**	$CONH_2$	$C_{17}H_{24}N_2O_3$	161–162	6.74 ± 0.24
8	**2i**	$CSNH_2$	$C_{17}H_{24}N_2O_2S$	135–136	6.81 ± 0.24 (4)
9	**2j**	$COCONH_2$	$C_{18}H_{24}N_2O_4$	107–109	6.8 ± 0.17
10	**2k**	CN	$C_{17}H_{22}N_2O_2$	61–61	7.05 ± 0.06 (4)
11	**2l**	H	$C_{16}H_{23}NO_2{\cdot}C_2H_2O_4$	141–142	5.23 ± 0.07
12	**2m**	CO_2Et	$C_{19}H_{27}NO_4$	oil	7.15 ± 0.15
13	**2n**	CO_2nPr	$C_{20}H_{29}NO_4$	oil	7.34 ± 0.1
14	**2o**	CO_2iPr	$C_{20}H_{29}NO_4$	oil	7.19 ± 0.09 (4)
15	**2p**	CO_2tBu	$C_{21}H_{31}NO_4$	oil	7.52 ± 0.16
16	**2q**	CO_2Ph	$C_{23}H_{27}NO_4$	oil	7.47 ± 0.3 (4)
17	**2r**	CO_2Bn	$C_{24}H_{29}NO_4$	oil	7.22 ± 0.2 (4)
18	**2s**	CONHMe	$C_{18}H_{26}N_2O_3$	oil	6.12 ± 0.09
19	**2t**	CONH(iPr)	$C_{20}H_{30}N_2O_3$	88–89	6.13 ± 0.14 (4)
20	**2u**	CONHPh	$C_{23}H_{28}N_2O_3$	127–128	6.37 ± 0.13
21	**2v**	CONHBn	$C_{24}H_{29}N_23O_3$	111–112	6.8 ± 0.17
22	**2w**	$CONMe_2$	$C_{19}H_{28}N_2O_3$	62–63	6.84 ± 0.15
23	**2x**	COnPr	$C_{20}H_{29}NO_3$	oil	6.57 ± 0.12
24	**2y**	COiPr	$C_{20}H_{29}NO_3$	oil	6.5 ± 0.04
25	**2z**	COPh	$C_{23}H_{27}NO_3$	109–111	7.17 ± 0.12
26	**2aa**	CO(2-thienyl)	$C_{21}H_{25}NO_3S$	104–106	7.15 ± 0.16 (4)
27	**2bb**	CO(2-indolyl)	$C_{25}H_{28}N_2O_3$	176–177	6.74 ± 0.1 (4)
28	**2cc**	CO(1-Me-2-pyrrolyl)	$C_{21}H_{28}N_2O_3$	oil	7.25 ± 0.26 (4)
29	**2dd**	CO(2,3-di-Me-4-isoxazolyl)	$C_{22}H_{28}N_2O_4$	oil	6.1 ± 0.2 (4)
30	**2ee**	(*R*)-CO_2Me	$C_{18}H_{25}NO_4$	81–82	6.73 ± 0.25 (5)
31	**2ff**	(*S*)-CO_2Me	$C_{18}H_{25}NO_4$	81–83	7.23 ± 0.07
32	**2gg**	(*R*)-$CONH_2$	$C_{17}H_{24}N_2O_3$	141–143	7 ± 0.02
33	**2hh**	(*S*)-$CONH_2$	$C_{17}H_{24}N_2O_3$	140–142	6.37 ± 0.13
34	1 (rolipram)		$C_{16}H_{21}NO_3$	131–132	6.65 ± 0.24 (9)

[a] All compounds are racemic except where designated. [b] Analytical data for all compounds (C, H, N analyses) were within ±0.4% of the theoretical values except entry 28 for which the following was obtained. C: calcd, 72.5; found, 71.9. [c] $pK_i = -\log K_i$ (M). [d] SD is the standard deviation of pK_i. [e] n is the number of experiments. In all cases $n = 3$ unless otherwise designated.

Table 2. Phosphodiesterase Enzyme Selectivity Data on a Representative Carbamate, Urea, and Amide of Pyrrolidine **2**[a,b]



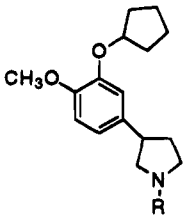
		% inh at 10 μm				% inh at 50 μM	
compd no.	R	I(+ Ca^{2+}/calm)	I (− Ca^{2+}/calm)	II (+ cGMP)	II (− cGMP)	III	V
2d	CO_2Me	4	0	16	39	5	57
2h	$CONH_2$	46	4	11	32	0	50
2c	COMe	2	0	15	26	0	36
1 (rolipram)			8		5	14[c]	

[a] Concentrations of Ca^{2+}/calmodulin and cGMP used in preparations are given in the Experimental Section. [b] A single experiment was conducted in duplicate to obtain the percent inhibition values. [c] Tested at 10 μM.

S-enantiomer. The difference in the SAR of the carbamate versus the urea of pyrrolidines coupled with their opposite stereospecificities of inhibition is noteworthy, yet not understood.

Three of the compounds from Table 1, **2c,d,h**, representing three different structural classes, amides, carbamates, and ureas, were tested for PDE family selectivity, cellular activity, and in vivo efficacy (Tables 2

Table 3. Cellular and in vivo Data on a Representative Carbamate, Urea, and Amide of Pyrrolidine **2**



compd no.	R	EC_{50} for TNF-α inh[a] ± SEM[b] (nm) (n)[c]	% inh of serum TNF-α ± SEM[b] (n)[c] 3 mg/kg po[d]	10 mg/kg po[d]
2d	CO_2Me	435 ± 204 (4)	32 ± 6 (3)	46 ± 4 (4)
2h	$CONH_2$	504 ± 138 (4)	51 ± 9 (3)	68 ± 1 (2)
2c	COMe	356 ± 130 (4)	7 ± 19 (2)	23 ± 8 (3)
1 (rolipram)		320 ± 204 (10)	55 ± 16 (2)	68 ± 8 (10)

[a] EC_{50} values for TNF-α inhibition in lipopolysaccharide-stimulated human monocytes. [b] SEM is the standard error of the mean. [c] Number of experiments. [d] Percent inhibition of serum TNF-α from lipopolysaccharide-injected mice.

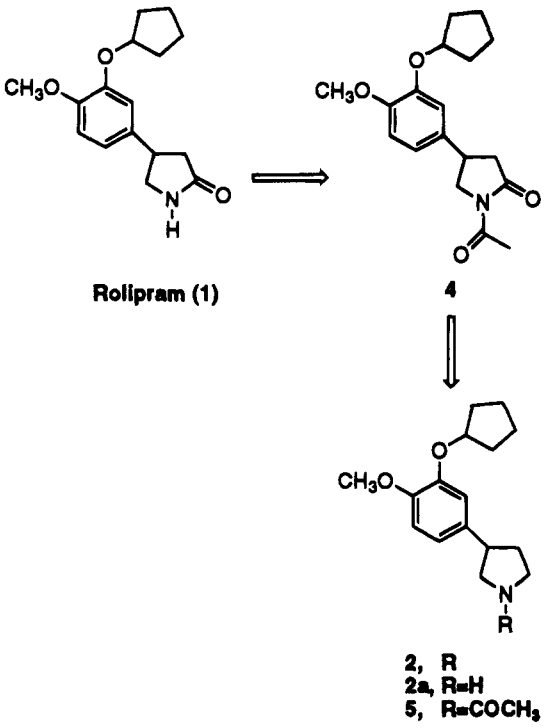


Figure 1. Progression from rolipram to pyrrolidines **2**.

and 3). All three compounds showed very good selectivity, approximately 50-fold or better, for inhibiting PDE-IV versus PDEs I–III and V. See Table 2. The same three compounds were also tested for their ability to inhibit LPS-induced TNF-α secretion from purified human peripheral blood monocytes. All three compounds demonstrated functional activity with IC_{50}s below 1 μM. In order to evaluate the potential of these compounds as anti-inflammatory agents, they were tested orally for their ability to reduce murine serum TNF-α levels. An initial in vitro experiment using an LPS-induced mouse peritoneal macrophage TNF-α production assay was conducted and confirmed that all three pyrrolidines and rolipram were active in the murine system (>65% inhibition at 1 μM). When tested in vivo, all three of the compounds inhibited serum TNF-α production in a dose-responsive manner. The potency of inhibition for amide **2h** (ED_{50} approximately 3 mg/kg) was equivalent to that of rolipram (Table 3).

In summary, we have synthesized a new class of PDE-IV inhibitors based on the prototype inhibitor rolipram. In general, this class of compounds demonstrates equivalent or greater potency at inhibiting PDE-IV relative to rolipram. The best PDE-IV inhibitors, **2p,q**, are approximately 10-fold more potent at inhibiting human PDE-IV than rolipram. Furthermore, three representative inhibitors, **2c,d,h**, of the amide, carbamate, and urea structural classes (Table 1) demonstrated good, dose-responsive functional activity in vitro and in vivo (po). The functional activity manifested by this class of inhibitors makes them promising medicinal chemistry lead structures for treating acute and chronic inflammatory disorders via PDE-IV inhibition.

Experimental Section

Representative examples of experimental procedures used to synthesize all of the compounds listed in Table 1 are given. All of the final synthesized products were characterized by obtaining melting points when appropriate (uncorrected), ^1H NMR spectra, and C, H, N elemental analyses. ^1H NMR spectra were determined using a Varian spectrometer (300 MHz superconducting, FT instrument), with $CDCl_3$ as the solvent, and are expressed in ppm downfield from internal tetramethylsilane. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. Reagents were used as received from commercial suppliers. Concentration of the reaction mixtures was performed by using a rotary evaporator at approximately water aspirator pressures. Flash silica gel chromatography was conducted with 230–400 mesh EM Science silica gel.

1-Formyl-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (2b). To 3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2a**) (330 mg, 1.26 mmol) was added ethyl formate. The solution was heated at 48 °C for 24 h. The volatiles were removed, and the residue was chromatographed on silica gel (1/1 hexanes/EtOAc) to give 1-formyl-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2b**) as a clear, colorless oil: yield 243 mg (67%). Anal. ($C_{17}H_{23}NO_3$) C, H, N.

1-Acetyl-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (2c). To a 23 °C solution of **2a** (295 mg, 1.13 mmol) in acetonitrile (4 mL) were added acetic anhydride (0.16 mL, 1.69 mmol, 150 mol %) and TEA (0.24 mL, 1.69 mmol, 150 mol %). The reaction mixture was stirred at 23 °C for 1 h. The reaction mixture was diluted with 1 M H_3PO_4 and EtOAc. The organics were washed with 1 M H_3PO_4, saturated $NaHCO_3$, and brine. The organics were dried over $MgSO_4$ and then concentrated. The residue was chromatographed on silica gel (EtOAc) to give 1-acetyl-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2c**) as a solid: yield 115 mg (34%); mp 58–60 °C. Anal. ($C_{18}H_{25}NO_3$) C, H, N.

1-Ureido-3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (2h). To a 23 °C solution of **2a** (730 mg, 2.79 mmol) and

4-(dimethylamino)pyridine (380 mg, 3.11 mmol, 110 mol %) in dichloromethane (CH_2Cl_2; 5 mL) was added trimethylsilyl isocyanate (0.37 mL, 2.73 mmol, 98 mol %). The reaction was stirred at 23 °C for 18 h. The reaction mixture was diluted with 1 M H_3PO_4 and CH_2Cl_2. The organics were washed with brine and then dried ($MgSO_4$) and concentrated to give a solid. The solid was recrystallized from EtOAc to give 1-ureido-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2h**) as a white solid: yield 297 mg (35%); mp 156–157 °C. Anal. ($C_{17}H_{24}N_2O_3$) C, H, N.

1-Cyano-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (2k). To a 23 °C solution of **2a** (570 mg, 2.18 mmol) and K_2CO_3 (452 mg, 3.27 mmol, 150 mol %) in acetonitrile (5 mL) was added CNBr (277 mg, 2.62 mmol, 120 mol %), and the reaction mixture was stirred for 16 h. The reaction mixture was diluted with water and EtOAc. The organics were washed with brine and then dried ($MgSO_4$) and concentrated to give an oil. The oil was chromatographed on silica gel (3/1 hexanes/EtOAc), and upon concentration 1-cyano-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2k**) was isolated as a solid: yield 240 mg (38%); mp 61–62 °C. Anal. ($C_{17}H_{22}N_2O_2$) C, H, N.

1-(Phenylureido)-3-(3-cyclopentoxy-4-methoxyphenyl)-pyrrolidine (2u). To a 0 °C solution of **2a** (300 mg, 1.15 mmol) in CH_2Cl_2 (2 mL) was added phenyl isocyanate (0.12 mL, 1.15 mmol, 100 mol %). The reaction mixture was stirred at 0 °C for 1 h and then diluted with H_2O and extracted with CH_2Cl_2. The combined organics were washed with 20% aqueous NH_4OH. The organics were then dried ($MgSO_4$) and concentrated, and the residue was chromatographed on silica gel (1/1 hexanes/EtOAc) to give 1-(phenylureido)-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2u**) as an oil which solidified to a white solid upon trituration with hexanes: yield 70 mg (39%); mp 127–128 °C. Anal. ($C_{23}H_{28}N_2O_3$) C, H, N.

1-(*N'*,*N'*-Dimethylureido)-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (2w). To a 0 °C solution of **2a** (200 mg, 0.765 mmol) and dimethylcarbamoyl chloride (70 μL, 0.765 mmol, 100 mol %) in CH_2Cl_2 (3 mL) was added TEA (0.2 mL, 1.53 mmol, 200 mol %). The reaction mixture was stirred for 1 h and then diluted with CH_2Cl_2 and 1 N NaOH. The organics were washed with 1 N NaOH, 1 M H_3PO_4, and brine. The organics were dried ($MgSO_4$) concentrated, and then chromatographed on silica gel (EtOAc) to give 1-(*N'*,*N'*-dimethylureido)-3-(3-cyclopentoxy-4-methoxyphenyl)pyrrolidine (**2w**) as an oil which solidified upon standing: yield 125 mg (49%); mp 62–63 °C. Anal. ($C_{19}H_{28}N_2O_3$) C, H, N.

PDE Type IVb Activity Assay and Assay for PDE Inhibition. Yeast-expressed PDE type IVb was assayed using a modified version of the coupled enzyme protocol previously described.[16] In this assay, the PDE type IVb hydrolyzes [^{3}H]-cAMP to [^{3}H]AMP. The [^{3}H]AMP is subsequently converted to [^{3}H]adenosine by excess 5′-nucleotidase. The amount of [^{3}H]-adenosine liberated is directly proportional to the hrPDE-IV activity. [^{14}C]Adenosine is used as an internal control. This assay is performed at 23 °C in a 100 μL reaction mixture containing 1 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM DTT, 0.05% *n*-octyl β-D-glucopyranoside, 0.5 mg/mL BSA, 1 μg of 5′-nucleotidase, 0.1 μM [^{3}H]cAMP, 1 μM [^{14}C]-adenosine, 50 nM PDE type IVb stock solution, and the desired concentration of test compound. Isotype specificity assays used either canine lung PDE-I ± 2 mM $CaCl_2$ and 1 μM calmodulin, PDE-II ± 10 μM cGMP, PDE-III, or PDE-V at 50 nM in place of yeast-expressed PDE type IVb. The PDE reactions were stopped with 200 μL of a slurry containing 50% Sephadex A-25 and 5 mM CAPS (pH 10). The mixture of [^{3}H]adenosine and [^{14}C]adenosine was eluted batchwise from the slurry, the amount of radioactivity was determined, and the PDE type IVb activity was calculated. Isotype specificities are expressed as percent inhibition against each canine enzyme and are set forth in Table 2.

The pK_is and $-\log K_i$s (M) of compounds set forth in the Tables were determined by measuring the inhibition, inh, of cAMP hydrolysis as a function of the concentration of the test compound, [D], over the range of 0.5 nM–100 μM. Each compound was tested at least three times, and the average and the standard deviation of the pK_is of the molar dissociation constant are set forth in the Table 1.

$$\text{inh} = \text{inh}_{\max} \times \frac{[D]}{K_i + [D]} + \text{inh}_{\min}$$

The type of inhibition (competitive, noncompetitive, or uncompetitive) was determined for the subset of compounds **2c**,**p**,**ff** by measuring the rate of cAMP hydrolysis as a function of both the concentration of the test compound (0, 3 × K_i, 10 × K_i, and 30 × K_i) and the concentration of cAMP (0.1 × K_m, 1 × K_m, 10 × K_m, and 50 × K_m, where the K_m = 2.75 μM for yeast-expressed PDE type IVb). The data were fit to each of the models shown below using RS/1 (BBN Software Products), and in all cases, the compounds showed strictly competitive behavior.

$$\text{competitive: rate} = \frac{V_{\max} \times \frac{[S]}{K_m}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}}$$

$$\text{noncompetitive: rate} = \frac{V_{\max} \times \frac{[S]}{K_m}}{\left(1 + \frac{[S]}{K_m}\right) \times \left(1 + \frac{[I]}{K_i}\right)}$$

$$\text{uncompetitive: rate} = \frac{V_{\max} \times \frac{[S]}{K_m}}{\frac{[S]}{K_m} \times \left(1 + \frac{[I]}{K_i}\right) + 1}$$

Assay for Inhibition of TNF-α Release from Murine Macrophages. C3H/HeJ female mice were injected ip with 2 mL of 5 mM $NaIO_4$. Five days later, mice were euthanized and peritoneal exudate cells collected into cold PBS. Erythrocytes were lysed with ammonium chloride if necessary. Cells were plated at 5 × 10^5 cells/well in a 24-well tissue culture plate and allowed to adhere for 1.5 h. Nonadherent cells were removed, and test PDE-IV inhibitors were added in RPMI medium with 1% fetal bovine serum. LPS (*Escherichia coli* serotype 0111:B4, Sigma Chemical Co.; 2.5 ng/mL final concentration) was then added, and cultures were incubated for 24 h at 37 °C. TNF-α protein was quantified in the supernatant fluids by a commercial TNF-α ELISA kit (Genzyme Co.).

Assay for Inhibition of TNF-α Release from Human Peripheral Blood Monocytes. Mononuclear cells were separated from heparinized venous blood from normal volunteers by Ficoll-Hypaque density centrifugation. Adhesion to plastic (96-well microtiter plates) was used to enrich for monocytes (80–85% purity). Cells were cultured under standard conditions in RPMI 1640 medium containing 2.5% heat-inactivated pooled human serum (Gibco). Compounds were added 15 min prior to stimulation of cells with LPS (10 ng/mL). Supernatants were collected 16 h later, and TNF-α was quantified with the help of commercial ELISA kits (Cistron) for human TNF-α.

Assay for Inhibition of Serum TNF-α Levels in Mice. Female C3H/Hen mice, 20–25 g body weight, were fasted overnight in preparation for oral dosing. Test compounds were suspended in 0.01% methyl cellulose and ground in a tissue homogenizer. Compounds were dosed 30 min prior to LPS injection. Five micrograms of LPS was then injected intraperitoneally into each mouse. Exactly 90 min after LPS injection, mice were bled from the heart. Blood was allowed to clot overnight at 4 °C. Samples were centrifuged for 10 min in a microcentrifuge, and the serum was removed and stored at −20 °C until analysis. Serum levels of TNF-α were subsequently measured using a commercially available mouse TNF-α ELISA kit (Genzyme), diluting the sample 1:5 and following the protocol enclosed in the kit. The percent inhibition of serum TNF-α levels caused by the compound was determined relative to serum TNF-α levels in control mice receiving vehicle alone.

Acknowledgment. We would like to thank Richard Silverstein and Tammy Stone for their assistance in the serum TNF-α assay, Manon Villeneuve for performing the chiral HPLC studies, and Drs. Donald Karanewsky and Rayomand Unwalla for helpful discussions. We also wish to thank Drs. Ken Ferguson, Vince Florio, Kate Loughney, and Tim Martins of the ICOS Corp., Dr. Graeme Bolger of the University of Utah, and Bart Steiner and Lothar Uher of ICOS for advice and reagents.

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JM940804N