Palladium-Catalyzed Cross-Coupling Reactions for the Synthesis of 6,8-Disubstituted 1,7-Naphthyridines: A Novel Class of Potent and Selective Phosphodiesterase Type 4D Inhibitors

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Recently, four subtypes of the human phosphodiesterase type 4 (PDE4A−D) enzyme have been described. So far, only very few PDE4 subtype-selective inhibitors are known. Herein, we describe the synthesis of 6,8-disubstituted 1,7-naphthyridines and their characterization as potent and selective inhibitors of PDE4D which suppress the oxidative burst in human eosinophils with IC_{50} values as low as 0.7 nM. SAR development and the extended use of palladium-catalyzed cross-coupling reactions led to compound **11** which inhibited human PDE4D with an IC_{50} value of 1 nM. Thus, compound **11** was 55, 175, and 1000 times more potent in inhibiting PDE4D over PDE4B, PDE4A, and PDE4C. In a Brown Norway rat model of allergic asthma, compound **11** when given by the oral route (1 mg/kg) reduced by more than 50% the influx of eosinophils, T-cells, and neutrophils into bronchoalveolar lavage fluid (BALF) samples obtained from antigen-challenged animals. Thus, PDE4D-selective inhibitors of the 1,7-naphthyridine class have the potential as an oral therapy for treating asthma.

Introduction

Modulation of the intracellular second messenger cyclic 3′,5′-adenosine monophosphate (cAMP) by phosphodiesterase type 4 (PDE4) inhibitors represents a promising new approach for the treatment of chronic inflammatory diseases such as asthma, COPD, and rheumatoid arthritis.[1,2] Originally fuelled mainly by preclinical findings, this approach has received substantial support from recent clinical studies, and the search for novel PDE4 inhibitors is currently one of the most active fields in the pharmaceutical industry.[3] PDE4 belongs to a family of at least seven structurally, biochemically, and pharmacologically distinct isoenzymes that catalyze the hydrolysis of cAMP or 3′,5′-guanosine monophosphate (cGMP) to the corresponding inactive 5′-nucleotide products.[4] Predominantly located in lymphocytes, monocytes, macrophages, neutrophils, eosinophils, and mast cells, but also in endothelial cells and epithelial cells, PDE4 seems to be the most important member of the PDE family in regulating immune and inflammatory responses.[1,5] Recently, different isogenes and splice variants of human PDE4 have been described.[4] The four gene products (PDE4A−D) are characterized by their selective, high-affinity hydrolysis of cAMP and sensitivity to inhibition by rolipram. According to an analysis of a number of human peripheral blood cells and cell lines, mRNAs of PDE4A,B,D are expressed in the majority of cell populations of the immune system.[5] In particular, PDE4D was prominently expressed in eosinophils, whereas PDE4C mRNA was not detected in any of the tested inflammatory cell lines. Nevertheless, the functional significance of the diverse cellular distribution of PDE4 subtypes remains to be established. The most prominent side effects related to the use of PDE4 inhibitors are nausea and emesis.[1,6] In addition, there is still concern about other potential untoward effects such as immunosuppression, metabolic disturbances, increased gastric acid secretion, and effects on the central nervous system.[1] Although the mechanism related to the induction of nausea and emesis is not fully understood, one hypothesis is that binding of inhibitors to the so-called rolipram high-affinity binding site is responsible for these effects so that potent PDE4 inhibitors with relatively low affinity for the rolipram high-affinity binding site should display good antiinflammatory activity with a decreased potential for side effects. Apparently, SB207499 (Ariflo; Chart 1), a PDE4 inhibitor currently in phase III clinical trials for the indications asthma and COPD, was selected based on an optimized ratio of PDE4 catalytic activity relative to affinity to the rolipram high-affinity binding site.[7] However, in clinical trials SB207499 still induced emesis at the first and/or second doses, albeit this effect apparently disappeared with continued treatment.[8] Thus, prediction of emesis in humans based on a relatively low ratio of PDE4 inhibition versus rolipram binding remains unsatisfactory.

Our research efforts in finding novel PDE4 inhibitors were based upon a different approach. We wanted to find orally active, second-generation PDE4 inhibitors displaying selectivity for one of the subtypes of human PDE4, in our case PDE4D, and thereby answer the question if such compounds retain the broad antiinflammatory properties of 'universal', non-PDE4 subtype-selective inhibitors. Furthermore, one might argue that, at least conceptually, PDE4 subtype-specific inhibitors may have a more favorable side effect profile than the current 'universal' PDE4 isozyme-selective compounds.[9] Indeed, SB207499, the most advanced PDE4 inhibitor

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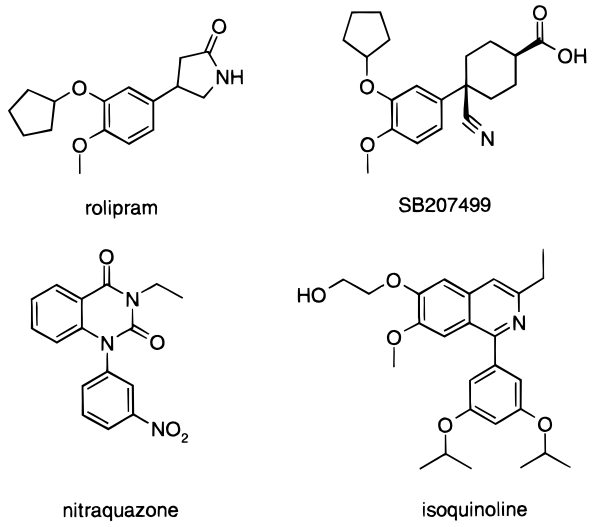
Chart 1



in clinical trials, is one of the very few compounds which is claimed to be moderately selective toward inhibition of PDE4D (relative to PDE4A−C).[2,7] PDE4 subtype-selective compounds could also serve as useful tools to answer the still open question of the functional significance of the diverse cellular distribution of PDE4 subtypes.

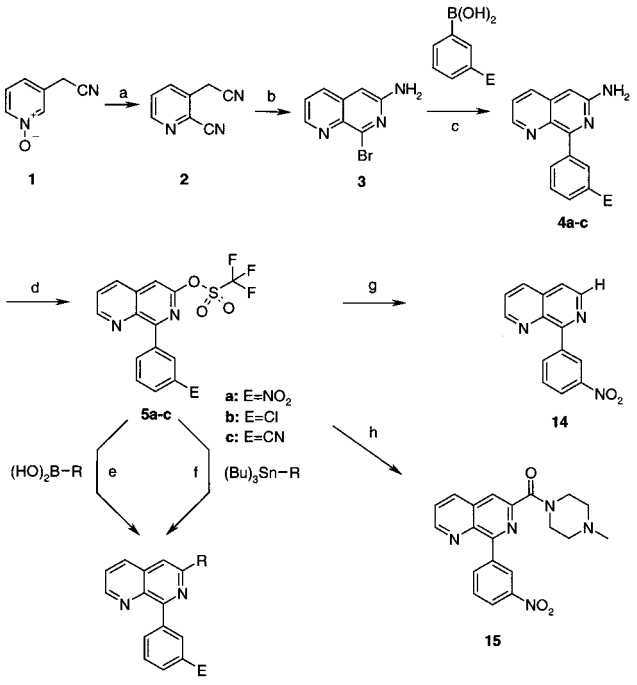
The majority of the currently described PDE4 inhibitors are structural variants of rolipram and contain the characteristic 3,4-dialkoxybenzyl substructure (Chart 1). Xanthine-type molecules form another class of PDE4 inhibitors. Alternatively, nitrogen-containing bicyclic aromatic ring systems, e.g. quinolines, heteroquinolines, or nitraquazones, emerged as very interesting 'templates' for PDE4 inhibitors. Isoquinolines represent another novel potent type of PDE4-selective inhibitor.[10] Recent reviews summarize the currently known different chemical classes of selective PDE4 inhibitors.[3]

Herein, we describe the discovery of 1,7-naphthyridines as a novel class of potent and PDE4D subtype-selective inhibitors. Palladium-catalyzed cross-coupling reactions[11] were used as powerful synthetic tools for the preparation of a variety of 6,8-disubstituted 1,7-naphthyridines starting from a common, appropriately functionalized intermediate. We report the SAR of these compounds with respect to PDE4A−D inhibition. Since eosinophils are believed to be key effector cells in the pathogenesis of asthma,[12] inhibition of the fMLP-induced oxidative burst in human eosinophils was used to evaluate the cellular activity of these PDE4D-selective compounds. Finally, we report data for the most potent compounds in an in vivo model of allergic asthma.

Results and Discussion

Chemistry. The synthesis of the key intermediate **3** is shown in Scheme 1. 2-Cyano-3-pyridylacetonitrile (**2**) was obtained from the *N*-oxide **1** by a modified Reissert−Heinze reaction.[13] This procedure turned out to be superior to the previously described synthesis[14] and led to regioselective cyanation at position 2 of the pyridine ring. Cyclization of dinitrile **2** following a modified procedure[15] with hydrogen bromide in acetic acid, i.e. circumventing the use of gaseous hydrogen

Scheme 1[a]



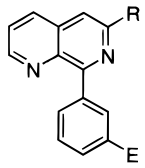
6: R=phenyl; E=NO_2
7: R=3'-pyridyl; E=NO_2
8: R=2'-furyl; E=NO_2
9: R=2'-tolyl; E=NO_2
10: R=allyl; E=NO_2
11: R=4'-carboxyphenyl; E=NO_2
12: R=4'-carboxyphenyl; E=Cl
13: R=4'-carboxyphenyl; E=CN

[a] Reagents and conditions: (a) $(CH_3)_3SiCN$, then diethylcarbamyl chloride, toluene, 60 °C; (b) HBr in acetic acid (33%), 25 °C; (c) $Pd(dba)_2$, $(C_6H_5)_3P$, K_2CO_3, DMF/H_2O/toluene, 100 °C; (d) $NaNO_2$, CF_3SO_3H, DMF, 25 °C; (e) $Pd(dba)_2$, $(C_6H_5)_3P$, 2 N Na_2CO_3 or 2 N K_2CO_3, THF or DMF, 80 °C; (f) $Pd(dba)_2$, $(C_6H_5)_3P$, LiCl, DMF, 80−100 °C; (g) $(C_2H_5)_3SiH$, [1,1′-bis(diphenylphosphino)-ferrocene]dichloropalladium(ll), DMF, 60 °C; (h) 1-methylpiperazine, CO, $(C_2H_5)_3N$, $Pd(dba)_2$, DMF, 80 °C.

bromide, gave intermediate **3**. Subsequent attachment of 3-substitued phenyl rings to position 8 of the 1,7-naphthyridine by Suzuki cross-coupling reaction led to compounds **4a**−**c**. Endeavors to convert the amino group into a leaving group at position 6 of the 1,7-naphthyridine which would allow us to apply further palladium-catalyzed cross-coupling reactions were less straightforward. All attempts to replace the 6-amino group by an iodo, bromo, or chloro atom via a Sandmeyer-type reaction failed. However, using similar reaction conditions and trifluoromethanesulfonic acid as a cosolvent allowed us to directly transform amines **4a**−**c** into the corresponding triflates **5a**−**c**. Subsequent carbon−carbon bond formation either via Suzuki or Stille-type reactions then led to the final products **6**−**13.** The nonsubstituted compound **14** was prepared via palladium-catalyzed reduction of triflate **5a** using triethylsilane as reagent.[16] Finally, palladium-catalyzed carbonylation of triflate **5a** in the presence of 4-methylpiperidine as a nucleophile led to the amide **15**.[17]

Structure−Activity Relationships and Biology. Most of the SAR around position 6 of the 1,7-naphthyridine was established with the 3-nitrophenyl moiety at position 8 (Table 1), since we quickly realized that an appropriately meta-substituted phenyl ring at this position was a prerequisite for obtaining good PDEA−D inhibitory activity. Other groups at position 8 such as phenyl, benzyl, naphthalene, heteroaromatic rings (e.g. furan-2-yl, thiophen-2-yl or benzothiophen-2-yl), or non-

Table 1. Inhibition of PDE4A−D and Subtype Selectivity Profile



			IC$_{50}$ (μM)[a]				
compd	R	E	PDE4A	PDE4B	PDE4C	PDE4D	ratio[b] 4B/4D
4a	NH_2	NO_2	>10	4.6(±1.2)	>10	1.6(±0.8)	2.9
5a	OSO_2CF_3	NO_2	1.8(±0.6)	0.31(±0.08)	3.2(±0.9)	0.011(±0.003)	28
5b	OSO_2CF_3	Cl	1.4	0.46	4.3	0.048	9.6
5c	OSO_2CF_3	CN	0.77(±0.31)	0.42(±0.12)	6.4(±2.5)	0.072(±0.046)	5.8
6	phenyl	NO_2	0.35	0.11(±0.03)	2.0	0.002(±0.001)	55
7	3-pyridyl	NO_2	0.12(±0.09)	0.081(±0.012)	0.61(±0.17)	0.003(±0.001)	27
8	2-furanyl	NO_2	9.5	0.51	>10	0.008(±0.0002)	64
9	2-tolyl	NO_2	3.8(±0.8)	0.34(±0.07)	>10	0.070(±0.012)	4.9
10	allyl	NO_2	0.279(±0.007)	0.15(±0.06)	0.89(±0.16)	0.007	21
11	4-carboxyphenyl	NO_2	0.088(±0.014)	0.049(±0.007)	0.068(±0.009)	0.001(±0.0002)	49
12	4-carboxyphenyl	Cl	0.059(±0.005)	0.045(±0.004)	0.19(±0.045)	0.002(±0.0002)	23
13	4-carboxyphenyl	CN	0.14(±0.034)	0.085(±0.007)	0.57(±0.02)	0.004(±0.001)	21
14	H	NO_2	>10	8.8(±0.5)	>10	4.1(±2.3)	2.1
15	4-methylpiperazin-1-ylcarbonyl	NO_2	>10	9.3	>10	2.0	4.7
SB207499			0.41(±0.10)	0.31(±0.11)	0.84(±0.18)	0.079(±0.028)	3.9
rolipram[d]			1.4(±0.2)	2.7(±0.6)	7.0(±0.3)	0.39(±0.10)	3.6[c]

[a] Data indicated as IC$_{50}$ or mean IC$_{50}$ ± SEM (n = 2−5). [b] Selectivity ratio indicated as IC$_{50}$ value in inhibiting PDE4B or [c] PDE4A divided by IC$_{50}$ value in inhibiting PDE4D. [d] Racemic. No satisfactory analysis obtained for compounds **4c**, **13**, and **14** but products were sufficiently pure according to ^{1}H NMR, HRMS, and HPLC.

aromatic cyclic groups (e.g. morpholin-4-yl) gave significantly less active compounds, regardless of the nature of the 6-substituent (IC$_{50}$ > 1 μM for PDE4A−D, data not shown). Despite the optimized 8-(3-nitrophenyl)-1,7-naphthyridine scaffold, compounds containing a small group at position 6 such as **4a** and **14** were only active in the micromolar range in the PDE4A−D assays. As soon as larger groups were attached to this position, potency toward inhibition of PDE4B and, particularly, PDE4D increased dramatically. For instance, replacing the 6-hydrogen atom of compound **14** (PDE4D IC$_{50}$ = 4100 nM) by a phenyl ring (compound **6**, PDE4D IC$_{50}$ = 2 nM) increased the potency against PDE4D by 2000-fold. Remarkably, compound **6** was selective toward PDE4D being 55, 175, and 1000 times more potent on PDE4D than on PDE4B, PDE4A, and PDE4C, respectively. Aromatic substitution at position 6 was not a prerequisite for achieving potent and selective PDE4D inhibition. Thus, the allylic compound **10** (PDE4D IC$_{50}$ = 7 nM) was only 3.5 times less active in the PDE4D assay than compound **6**, albeit the selectivity toward PDE4B was decreased by 21-fold. Even the triflate **5a** was still very potent (PDE4D IC$_{50}$ = 11 nM), indicating that there is some degree of steric tolerance at position 6. On the other hand, the amide **15** was only weakly active (PDE4D IC$_{50}$ = 2000 nM) demonstrating the subtlety of the SAR around position 6. Heteroaromatic substitution at this position also gave very potent compounds (**7** and **8**) with similar selectivity profiles. A comparison of the IC$_{50}$ values of compounds **9** and **6** shows that the methyl group at the ortho position of the phenyl ring not only diminished inhibitory potency toward PDE4D (IC$_{50}$ = 70 nM versus 2 nM) but also almost completely abolished PDE4D/PDE4B selectivity (5-fold versus 55-fold). The benzoic acid-substituted 1,7-naphthyridine **11** was the most active compound found in our series. It selectively inhibited PDE4D (IC$_{50}$ = 1 nM) and was 49-, 68-, and 88-fold less potent on PDE4B, PDE4A, and PDE4C, respectively. Thus, compound **11** when compared to SB207499 was 80-fold more potent in inhibiting PDE4D and, particularly, had a significantly better selectivity profile toward the other PDE4 subtypes. As mentioned above, meta-substituted phenyl rings at position 8 of the 1,7-naphthyridine were a prerequisite for achieving potent PDE4D inhibitory activity. Particularly, electron-withdrawing substituents such as the nitro, cyano, or chloro group were best suited. A comparison of the 3′-nitrophenyl-substituted compounds **5a** and **11** with the corresponding 3′-chlorophenyl analogues (**5b**, **12**) and 3′-cyanophenyl analogues (**5c**, **13**) shows that 8-(3′-nitrophenyl)-substituted 1,7-naphthyridines were most potent in inhibiting PDE4D followed by the 8-(3′-chlorophenyl) and 8-(3′-cyanophenyl) analogues. The same rank order could be found with respect to their selectivity profile toward inhibition of PDE4A−D.

A selection of compounds was also tested against phosphodiesterase type 3 (PDE3) and for their ability to compete with the (rat) high-affinity [^{3}H]rolipram binding site (Table 2).[7,18] All molecules were completely inactive against PDE3. Against rolipram binding, the compounds measured were about 2-fold more potent in the rolipram binding assay than in the PDE4D enzyme assay. Thus, in terms of their ability to inhibit PDE4D catalytic activity versus their ability to compete for the high-affinity [^{3}H]rolipram binding site compounds **5a**, **6,** and **11**−**13** were not selective. Nevertheless, they had a very similar profile to SB207499 which, in our hands, was also about twice as potent in inhibiting [^{3}H]rolipram binding versus inhibiting PDE4D catalytic activity. In contrast and consistent with earlier findings,[7] the reference compound rolipram was significantly more potent in displacing [^{3}H]rolipram from its binding site than in inhibiting PDE4A−D (>125-fold).

Table 2. Inhibition of [^{3}H]Rolipram Binding, of fMLP-Induced Oxidative Burst in Human Eosinophils, and of PDE3

	IC_{50}		
compd	rolipram (nM)[a]	HUEOS (nM)[b]	PDE3 (μM)[c]
4a	–	41(±11)	–
5a	7.4	4(±4)	>100
6	4.0	10(±10)	>100
7	–	3(±3)	–
8	–	11(±12)	–
9	–	42(±13)	–
10	–	3(±3)	–
11	0.6(±0.2)	0.7(±0.4)	>100
12	0.8	–	>100
13	2.4(±0.8)	–	>100
14	58	548(±344)	–
15	–	–	>100
SB207499	40(±13)	–	>100
rolipram[d]	3.1(±0.3)	–	>100

[a] Data indicated as IC_{50} or mean IC_{50} ± SEM ($n = 3-4$). [b] Data indicated as mean IC_{50} ± SEM. For each compound three independent measurements were done using eosinophils from three different donors. [c] $n = 1-2$. [d] Racemic.

Table 3. Inhibition of OA-Induced Leukocyte Infiltration, Eosinophil Activation, and Protein Release in BALF from Actively Sensitized Brown Norway Rats

compd	dose[a] (mg/kg)	eosinoph[b]	EPO[c]	T-lymph[b]	neutroph[b]	protein[d]
5a	2 × 1	0	0	–	–	–
6	2 × 1	0	2	–	–	–
11	2 × 1	86 ± 4	92 ± 3	59 ± 8	71 ± 4	76 ± 4
12	2 × 1	51 ± 14	55 ± 13	23 ± 15	55 ± 6	49 ± 7
13	2 × 1	44 ± 13	92 ± 1	40 ± 16	21 ± 21	33 ± 14
SB207499	2 × 10	56 ± 10	41 ± 15	40 ± 9	64 ± 8	47 ± 5

[a] Since BALF is collected only 48 h after allergen challenge, all compounds were given twice (po), 1 h prior to and 24 h after allergen exposure. For more details, see Experimental Section. [b] Percentage inhibition of the cell numbers measured in BALF from allergen-challenged, drug-treated versus allergen-challenged, vehicle-treated (control) animals. The results obtained are the mean ± SEM of 5−10 animals/group. [c] Percentage inhibition of eosinophil activation measured as EPO activity recovered in BALF from allergen-challenged, drug-treated versus control animals. [d] Percentage inhibition on protein concentration recovered in BALF from allergen-challenged, drug-treated versus control animals.

A panel of compounds was tested for their inhibitory effect on the fMLP-induced oxidative burst in human peripheral blood eosinophils[19] (Table 2). This assay was used both to evaluate the propensity of the compounds to modulate a key effector cell involved in the pathology of asthma[12] and as a means to check the compounds' ability to penetrate cells. The assay was not used as a tool to develop SAR. The IC_{50} values obtained had a certain degree of variation since each compound was independently tested in eosinophils derived from blood from three different donors. This did not allow us to make a clear potency rank order in this cellular assay. Nevertheless, all compounds tested potently inhibited the oxidative burst of human eosinophils, and the benzoic acid-substituted 1,7-naphthyridine **11** (IC_{50} = 0.7 nM) was again among the most potent compounds.

To address the question as to whether our PDE4D-selective compounds had antiinflammatory properties in vivo, we tested the more promising molecules in the antigen-induced pulmonary eosinophilia rat model, an animal model of allergic asthma[20] (Table 3). Briefly, ovalbumin (OA)-sensitized Brown Norway rats were challenged with aerosolized antigen (OA). This resulted in an accumulation of eosinophils, T-lymphocytes, and neutrophils in BALF samples obtained from these animals. In addition, BALF samples also contained increased levels of both proteins and eosinophil granule-derived eosinophil peroxidase (EPO). These two proteins are markers for increased airway microvascular permeability[21] and for eosinophil activation at the site of inflammation, respectively. Whereas the triflate **5a** and the phenyl-substituted compound **6** showed no oral activity at all, the benzoic acid-substituted 1,7-naphthyridines **11**−**13** when given twice, 1 h before and 24 h after OA challenge (1 mg/kg), potently inhibited eosinophil influx and production of EPO. Although drug plasma levels were not measured, this finding indicates that the benzoic acid moiety was important to convey oral effect bioavailability. The reference compound SB207499 was also active in this model, albeit at a higher concentration and to a lesser extent. Consistent with its in vitro profile compound **11** showed the greatest activity and completely blocked eosinophil influx and EPO production. A deeper analysis of the BALF samples of animals treated with **11**−**13** revealed that additional markers of airway inflammation such as infiltration of T-lymphocytes and neutrophils and the release of proteins were also inhibited although to a smaller extent. Again, compound **11** showed the greatest effect on these parameters. Thus, the selective PDE4D inhibitors **11**−**13** showed potent antiinflammatory activity in an in vivo model of allergic asthma.

Conclusion

We have used palladium-catalyzed cross-coupling reactions to synthesize 6,8-disubstituted 1,7-naphthyridines which constitute a novel class of potent and selective inhibitors of the PDE4D. In vitro these compounds potently inhibited fMLP-induced oxidative burst in human eosinophils, a key effector cell in the pathogenesis of asthma. In contrast to the archetype PDE4 inhibitor rolipram, these PDE4D-selective compounds showed no preference for competing for the [^{3}H]rolipram binding site versus PDE4D catalytic activity and, in this respect, had a similar profile as the reference compound SB207499. It remains to be shown whether selectivity for PDE4D over PDE4A−C translates into a reduced risk of emesis in man. The benzoic acid-substituted 1,7-naphthyridines **11**−**13** were orally active in an animal model of asthma and potently inhibited inflammatory cell influx, eosinophil activation, and protein release into BALF from ovalbumin-challenged actively sensitized Brown Norway rats. Thus, PDE4D subtype-selective inhibitors from the 1,7-naphthyridine class have the potential as a novel oral therapy for the treatment of asthma.

Experimental Section

General. Melting points were measured with a Buechi B-540 and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker DPX-400 (400 MHz) or AM-360 (360 MHz) or a Varian Gemini-200 (200 MHz) instrument, using residual solvent protons as references. Mass spectra were recorded on a VG 70-SE instrument or a MAT 900 mass spectrometer (ESI) at nominal 6000 resolution. Flash chromatography was carried out using silica gel (Merck; Kieselgel 60 F_{254}, 230−400 mesh). Preparative thin-layer chromatography was done using silica gel 60 F_{254} PLC plates (Merck; 20 × 20, 0.5 mm) and analytical TLC was performed using precoated glass plates. AR grade solvents (Fluka) and commercial reagents (Fluka, Aldrich,

Lancaster) were used without further purification in all cases. *Brine* refers to a saturated aqueous NaCl solution and concentration procedures were carried out using a rotary evaporator at water aspirator pressure. Organic extracts were routinely dried over anhydrous Na_2SO_4. Pd-catalyzed reactions were run under an argon atmosphere in degassed solvents.

3-Cyanomethylpyridine-2-carbonitrile (2). To a stirred suspension of 3-cyanomethylpyridine *N*-oxide (10 g, 74.6 mmol) in toluene (60 mL) was added trimethylsilanecarbonitrile (9.0 g, 90.7 mmol). To this suspension was added dimethylcarbamyl chloride (8.0 g, 74.4 mmol) and the mixture was stirred for 20 h at 60 °C. The mixture was cooled and diluted with EtOAc and 1 N K_2CO_3. The layers were separated and the aqueous phase was extracted twice with EtOAc. The combined organic phases were dried and concentrated. Recrystallization of the crude product from ethanol gave 7.2 g (67%) of product **2**: mp 61−63 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.08 (s, 2H), 7.62 (dd, J = 5.0, 9.0 Hz, 1H), 8.05 (d, J = 9.0 Hz, 1H), 8.73 (d, J = 5.0 Hz, 1H). Anal. ($C_8H_5N_3$) C, H, N.

8-Bromo-[1,7]naphthyridin-6-ylamine (3). To a solution of 33% HBr in acetic acid (120 mL) was added in portions 3-cyanomethylpyridine-2-carbonitrile (**2**) (20 g, 140 mmol) so that the reaction temperature did not exceed 30−35 °C. The reaction mixture was stirred at ambient temperature for 1 h. The suspension was cooled in an ice bath, filtered, and washed with EtOAc. The solid was triturated in boiling MeOH (400 mL), cooled to ambient temperature, and filtered. The finely powdered material was taken up in water (400 mL) and saturated Na_2CO_3 added until pH 8 was reached. The suspension was vigorously stirred for 30 min and filtered to afford 23.1 g (74%) of the title compound **3**: mp 187 °C dec; ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.45 (s, broad, 2H), 6.62 (s, 1H), 7.50 (dd, J = 5.4, 8.4 Hz, 1H), 8.04 (d, J = 8.4 Hz, 1H), 8.62 (d, broad, J = 5.4 Hz, 1H). Anal. ($C_8H_6BrN_3$) C, H, Br, N.

General Procedure A. Suzuki Couplings of 8-Bromo-[1,7]naphthyridin-6-ylamine (3): 8-(3-Nitrophenyl)-[1,7]-naphthyridin-6-ylamine (4a). To a solution of **3** (5.0 g, 22.3 mmol) in toluene/DMF (1:1) (60 mL) were added bis(dibenzylideneacetone)palladium (256 mg, 0.44 mmol), triphenylphosphine (460 mg, 1.75 mmol), 3-nitrophenylboronic acid (3.91 g, 23.4 mmol), and K_2CO_3 (6.2 g, 44.9 mmol), dissolved in 15 mL of water. The mixture was stirred under reflux for 4 h. The reaction mixture was diluted with EtOAc, filtered through Celite, and washed with water. The organic layer was dried and concentrated. The solid was triturated in ether and filtered to afford 5.4 g of product (90%). An analytical sample was recrystallized from 2-propanol: mp 228−229 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 6.36 (s, 2H), 6.74 (s, 1H), 7.49 (dd, J = 4.2, 6.6 Hz, 1H), 7.80 (dd, J = 8.4, 8.4 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 8.33 (dd, J = 2.4, 8.4 Hz, 1H), 8.59 (d, J = 6.6 Hz, 1H), 8.62 (d, J = 4.2 Hz, 1H), 8.93 (d, J = 2.4 Hz, 1H); HRMS [M + H]$^+$ found 267.0881, calcd for $C_{14}H_{11}N_4O_2$ 267.0882. Anal. ($C_{14}H_{10}N_4O_2$) C, H, N, O.

8-(3-Chlorophenyl)-[1,7]naphthyridin-6-ylamine (4b). Purified by chromatography (10:1 toluene/acetone): white solid, 74% yield. Analytical sample recrystallized from 2-propanol: mp 148−149 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.28 (s, broad, 2H), 6.68 (s, 1H), 7.46 (dd, J = 5.4, 8.4 Hz, 1H), 7.48−7.55 (m, 2H), 7.99−8.10 (m, 3H), 8.58 (s, broad, 1H); HRMS [M + H]$^+$ found 256.0639, calcd for $C_{14}H_{11}ClN_3$ 256.0642. Anal. ($C_{14}H_{10}ClN_3 \cdot 0.15H_2O$) C, H, Cl, N.

3-(6-Amino-[1,7]naphthyridin-8-yl)benzonitrile (4c). Purified by trituration in ether: pale yellow solid, 90% yield. Analytical sample recrystallized from 2-propanol: mp 182−184 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.33 (s, broad, 2H), 6.72 (s, 1H), 7.48 (dd, J = 5.4, 8.4 Hz, 1H), 7.72(dd, J = 7.2, 7.2 Hz, 1H), 7.93 (d, J = 7.2 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 8.40 (d, J = 8.4 Hz, 1H), 8.46 (s, broad, 1H), 8.60 (d, broad, J = 5.4 Hz, 1H); HRMS [M + H]$^+$ found 247.0978, calcd for $C_{15}H_{11}N_4$ 247.0984.

General Procedure B. Synthesis of Trifluoromethanesulfonic Acid [1,7]Naphthyridin-6-yl Esters 5a−c: Trifluoromethanesulfonic Acid 8-(3-Nitrophenyl)-[1,7]naphthyridin-6-yl Ester (5a). To a solution of **4a** (4 g, 15.0 mmol) in a 2:1 mixture of DMF/trifluoromethanesulfonic acid (30 mL) was carefully added in several portions sodium nitrite (2.07 g, 30.0 mmol). The solution was stirred for 3 h at 25 °C. The reaction mixture was diluted with EtOAc and washed with water, 2 N NaOH, and water again. The organic phase was dried and concentrated. The solid was triturated in ether (120 mL) and filtered and the filtrate concentrated. The remaining residue was purified by chromatography (toluene) to afford 3.6 g of product (60%): mp 106−108 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.91 (dd, J = 8.4, 8.4 Hz, 1H), 7.99 (dd, J = 5.4, 7.2 Hz, 1H), 8.35 (s, 1H), 8.44 (d, broad, J = 8.4 Hz, 1H), 8.65 (d, J = 7.2 Hz, 1H), 8.69 (d, J = 8.4 Hz, 1H), 9.06 (s, broad, 1H), 9.25 (d, J = 5.4 Hz, 1H); MS (*m*/*e*) [M + H]$^+$ 400. Anal. ($C_{15}H_8F_3N_3O_5S$) C, H, F, N, O, S.

Trifluoromethanesulfonic Acid 8-(3-Chlorophenyl)-[1,7]naphthyridin-6-yl Ester (5b). Purified by chromatography (10:0.5 toluene/acetone): pale brown solid, 34% yield. Analytical sample recrystallized from 2-propanol: mp 96−97 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.45−7.51 (m, 2H), 7.55 (s, 1H), 7.70 (dd, J = 5.4, 9.0 Hz, 1H), 8.16−8.19 (m, 1H), 8.27 (d, broad, J = 9.0 Hz, 1H), 8.31 (s, broad, 1H), 9.13 (dd, J = 0.3, 5.4 Hz, 1H); HRMS [M + H]$^+$ found 388.9980, calcd for $C_{15}H_9ClF_3N_2O_3S$ 388.9981. Anal. ($C_{15}H_8ClF_3N_2O_3S$) C, H, Cl, F, N, O, S.

Trifluoromethanesulfonic Acid 8-(3-Cyanophenyl)-[1,7]naphthyridin-6-yl Ester (5c). Purified by chromatography (10:0.5 toluene/acetone): white solid, 50% yield; mp 102−104 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.66 (dd, J = 7.2, 7.2 Hz, 1H), 7.75 (dd, J = 5.4, 8.4 Hz, 1H), 7.81 (d, J = 7.2 Hz, 1H), 8.32 (d, broad, J = 8.4 Hz, 1H), 8.54 (d, J = 7.2 Hz, 1H), 7.74 (s, broad, 1H), 9.15 (dd, J = 1.8, 5.4 Hz, 1H); MS (*m*/*e*) [M + H]$^+$ 380. Anal. ($C_{16}H_8F_3N_3O_3S$) C, H, F, N, O, S.

General Procedure C. Stille Couplings of Trifluoromethanesulfonic Acid 8-(3-Nitrophenyl)-[1,7]naphthyridin-6-yl Ester (5a): 8-(3-Nitrophenyl)-6-pyridin-3-yl-[1,7]-naphthyridine (7). To a degassed solution of **5a** (150 mg, 0.375 mmol) in DMF (2 mL) were added bis(dibenzylideneacetone)palladium (4.5 mg, 0.008 mmol), triphenylphosphine (8 mg, 0.031 mmol), LiCl (48 mg, 1.13 mmol), and 3-tributylstannylpyridine (145 mg, 0.394 mmol). The reaction mixture was stirred at 110 °C for 16 h. The solution was diluted with EtOAc and water. A part of the product precipitated. The biphasic mixture was filtered and the residue thoroughly washed with water and dried at 50 °C under reduced pressure to give 64 mg of pure product. The organic layer of the filtrate was separated, washed with water, dried, and concentrated. The residue was triturated in ether and filtered to give additional 28 mg of pure product (75%, total yield). An analytical sample was recrystallized from acetonitrile: mp 247−248 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.64 (dd, J = 5.4, 8.4 Hz, 1H), 7.87−7.94 (m, 2H), 8.42 (d, broad, J = 7.2 Hz, 1H), 8.60 (d, J = 7.2 Hz, 1H), 8.64−8.79 (m, 4H), 9.15 (s, broad, 2H), 9.50 (s, broad, 1H); HRMS [M + H]$^+$ found 329.1042, calcd for $C_{19}H_{13}N_4O_2$ 329.1039. Anal. ($C_{19}H_{12}N_4O_2$) C, H, N, O.

6-Furan-2-yl-8-(3-nitrophenyl)-[1,7]naphthyridine (8). The reaction was finished after 3 h at 80 °C. Product purified by trituration in ether: pale yellow solid, 62% yield. Analytical sample recrystallized from 2-propanol: mp 202−203 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 6.60 (dd, J = 1.8, 4.8 Hz, 1H), 7.26 (d, J = 4.8 Hz, 1H), 7.59−7.65 (m, 2H), 7.71 (dd, J = 7.2, 7.2 Hz, 1H), 8.02 (s, 1H), 8.24 (d, broad, J = 7.2 Hz, 1H), 8.36 (d, broad, J = 7.2 Hz, 1H), 8.64 (d, J = 7.2 Hz, 1H), 9.00 (dd, J = 1.2, 5.4 Hz, 1H), 9.17 (s, broad, 1H); HRMS [M + H]$^+$ found 318.0883, calcd for $C_{18}H_{12}N_3O_3$ 318.0879. Anal. ($C_{18}H_{11}N_3O_3$) C, H, N, O.

6-Allyl-8-(3-nitrophenyl)-[1,7]naphthyridine (10). The reaction was finished after 2 h at 80 °C. Product purified by trituration in ether: pale yellow solid, 52% yield; mp 84−86 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 3.80 (d, J = 6.6 Hz, 2H), 5.18 (d, J = 18 Hz, 1H), 5.24 (d, J = 24 Hz, 1H), 6.15−6.28 (m, 1H), 7.78−7.87 (m, 3H), 8.36 (d, broad, J = 7.2 Hz, 1H), 8.49 (d, J = 7.2 Hz, 1H), 8.62 (d, J = 7.2 Hz, 1H), 8.99 (s,

broad, 1H), 9.05 (d, broad, J = 5.4 Hz, 1H); MS (*m/e*) $[M+H]^+$ 290. Anal. ($C_{17}H_{13}N_3O_3$) Calcd: C(70.09), H(4.50), N(14.42), O(10.98). Found: C(69.55), H(4.40), N(14.22), O(10.70).

General Procedure D. Suzuki Couplings of Trifluoromethanesulfonic Acid 8-(3-Nitrophenyl)-[1,7]naphthyridin-6-yl Ester (5a): 8-(3-Nitrophenyl)-6-phenyl-[1,7]naphthyridine (6). To a solution of **5a** (300 mg, 0.751 mmol) in THF (5 mL) and 2 N Na_2CO_3 (1.5 mL) were added bis(dibenzylideneacetone)palladium (17.3 mg, 0.030 mmol), triphenylphosphine (15.7 mg, 0.060 mmol), and phenylboronic acid (118 mg, 0.968 mmol). The reaction mixture was stirred at 80 °C for 16 h. The solution was diluted with EtOAc, filtered trough Celite, and washed with 1 N NaOH and brine. The organic layer was dried and concentrated to give 205 mg of pure product (84%). An analytical sample was recrystallized from 2-propanol: mp 177−178 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.45−7.59 (m, 3H), 7.65 (dd, J = 5.4, 8.4 Hz, 1H), 7.72 (dd, J = 7.2, 7.2 Hz, 1H), 8.13 (s, 1H), 8.23−8.31 (m, 3H), 8.36 (d, broad, J = 7.2 Hz, 1H), 8.70 (d, J = 7.2 Hz, 1H), 9.04 (d, broad, J = 5.4 Hz, 1H), 9.24 (s, broad, 1H); HRMS $[M + H]^+$ found 328.1088, calcd for $C_{20}H_{14}N_3O_2$ 328.1086; Anal. ($C_{20}H_{13}N_3O_2$) C, H, N, O.

8-(3-Nitrophenyl)-6-*o*-tolyl-[1,7]naphthyridine (9). Purified by trituration in ether: pale yellow solid, 54% yield. Analytical sample recrystallized from 2-propanol: mp 148−149 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 2.49 (s, 3H), 7.33−7.41 (m, 3H), 7.60−7.65 (m, 1H), 7.86 (dd, J = 7.2, 7.2 Hz, 1H), 7.88 (dd, J = 5.4, 8.4 Hz, 1H), 8.17 (s, 1H), 8.37 (d, broad, J = 8.4 Hz, 1H), 8.60 (d, broad, J = 8.4 Hz, 1H), 8.69 (d, J = 7.2 Hz, 1H), 9.07 (s, broad, 1H), 9.13 (d, broad, J = 5.4 Hz, 1H); HRMS $[M + H]^+$ found 342.1240, calcd for $C_{21}H_{16}N_3O_2$ 342.1243. Anal. ($C_{21}H_{15}N_3O_2 \cdot 0.05H_2O$) C, H, N, O.

4-[8-(3-Nitrophenyl)-[1,7]naphthyridin-6-yl]benzoic Acid (11). To a solution of **5a** (700 mg, 1.75 mmol) in DMF (10 mL) and 2 N Na_2CO_3 (5 mL) were added bis(dibenzylideneacetone)palladium (16 mg, 0.028 mmol), triphenylphosphine (29 mg, 0.111 mmol), and 4-carboxyphenylboronic acid (378 mg, 2.28 mmol). The reaction mixture was stirred at 80 °C for 2 h. The solution was diluted with EtOAc and 2 N HCl. The precipitated solid was filtered, thoroughly washed with water, and dried at 50 °C under reduced pressure to give 610 mg of pure product (94%). An analytical sample was recrystallized from formic acid: mp > 250 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.83−7.89 (m, 2H), 8.11 (d, J = 7.8 Hz, 2H), 8.36−8.42 (m, 3H), 8.58 (d, J = 7.2 Hz, 1H), 8.68−8.71 (m, 2H), 9.08−9.12 (m, 2H); HRMS $[M + H]^+$ found 372.0989, calcd for $C_{21}H_{14}N_3O_4$ 372.0984. Anal. ($C_{21}H_{13}N_3O_4 \cdot 0.1HCO_2H$) C, H, N, O.

4-[8-(3-Chlorophenyl)-[1,7]naphthyridin-6-yl]benzoic Acid (12). To a suspension of **5b** (1.66 g, 4.27 mmol) in DMF (17 mL) and 2 N Na_2CO_3 (12 mL) were added bis(dibenzylideneacetone)palladium (99 mg, 0.172 mmol), triphenylphosphine (73 mg, 0.278 mmol), and 4-carboxyphenylboronic acid (850 mg, 5.12 mmol). The reaction mixture was stirred at 80 °C for 3 h. The mixture was diluted with EtOAc and 2 N HCl. The biphasic solution was filtered through Celite; the organic layer was separated and washed with water and brine. The solution was concentrated and the precipitated product was filtered and dried to give 1.43 g of product (93%). An analytical sample was recrystallized from THF: mp > 250 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.57−7.64 (m, 2H), 7.85 (dd, J = 5.4, 9.0 Hz, 1H), 8.12 (d, J = 7.8 Hz, 2H), 8.13−8.21 (m, 1H), 8.26 (s, broad, 1H), 8.41 (d, J = 7.8 Hz, 2H), 8.56 (d, J = 9.0 Hz, 1H), 8.66 (s, 1H), 9.10 (d, broad, J = 5.4 Hz, 1H); MS (*m/e*) $[M]^+$ 360. Anal. ($C_{21}H_{13}ClN_2O_2$) C, H, Cl, N, O.

4-[8-(3-Cyanophenyl)-[1,7]naphthyridin-6-yl]benzoic Acid (13). To a suspension of **5c** (2.15 g, 5.67 mmol) in DMF (22 mL) and 2 N K_2CO_3 (17 mL) were added bis(dibenzylideneacetone)palladium (131 mg, 0.228 mmol), triphenylphosphine (95 mg, 0.363 mmol), and 4-carboxyphenylboronic acid (1.13 g, 6.81 mmol). The reaction mixture was stirred at 85 °C for 3 h. The hot solution was filtered through Celite and the filtrate was cooled in an ice bath. The precipitate was filtered, washed with EtOAc, and dried. The solid was dissolved at 80 °C in a 3:1 mixture water−DMF (40 mL) and the crude product was precipitated by carefully adding 2 N HCl (8 mL) and water (10 mL). The cold suspension was filtered and the crude product was purified by trituration in hot THF to give 1.12 g of pure product (56%). An analytical sample was recrystallized from formic acid/water: mp > 250 °C; ^{1}H NMR (360 MHz, DMSO-d_6) δ 7.78 (dd, J = 7.2, 7.2 Hz, 1H), 7.86 (dd, J = 5.4, 8.4 Hz, 1H), 8.00 (d, J = 7.2 Hz, 1H), 8.12 (d, J = 7.8 Hz, 2H), 8.41 (d, J = 7.8 Hz, 2H), 8.54 (d, J = 7.2 Hz, 1H), 8.58 (d, J = 7.2 Hz, 1H), 8.63 (s, 1H), 8.66 (s, 1H), 9.10 (s, broad 1H); HRMS $[M + H]^+$ found 352.1090, calcd for $C_{22}H_{14}N_3O_2$ 352.1086.

8-(3-Nitrophenyl)-[1,7]naphthyridine (14). To a degassed solution of **5a (**50 mg, 0.125 mmol) in DMF (1 mL) were added triethylsilane (36.4 mg, 0.313 mmol) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(ll) (2 mg, 0.0024 mmol). The mixture was stirred at 60 °C for 3 h. Then, [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(ll) (2 mg, 0.0024 mmol) was added again and the reaction mixture was stirred at 60 °C for an additional hour. The solution was diluted with EtOAc and washed with water. The organic layer was dried and concentrated. Purification by preparative thin-layer chromatography (5:1 toluene/acetone) gave 29 mg of pure product (92%). An analytical sample was recrystallized from 2-propanol: mp 182−183 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.67 (dd, J = 5.4, 8.4 Hz, 1H), 7.70 (dd, J = 7.2, 7.2 Hz, 1H), 7.73 (d, J = 5.4 Hz, 1H), 8.27 (d, J = 7.2 Hz, 1H), 8.36 (d, broad, J = 8.4 Hz, 1H), 8.55 (d, broad, J = 7.2 Hz, 1H), 8.77 (d, J = 5.4 Hz, 1H), 9.08−9.12 (m, 2H); HRMS $[M + H]^+$ found 252.0771, calcd for $C_{14}H_{10}N_3O_2$ 252.0773.

(4-Methylpiperazin-1-yl)[8-(3-nitrophenyl)-[1,7]naphthyridin-6-yl]methanone (15). To a degassed solution of **5a (**150 mg, 0.376 mmol) in DMF (6 mL) were added bis(dibenzylideneacetone)palladium (4.2 mg, 0.007 mmol), triphenylphosphine (7.8 mg, 0.030 mmol), triethylamine (76 mg, 0.750 mmol), and 1-methylpiperazine (41 mg, 0.401 mmol). The reaction mixture was stirred at 80 °C under a CO atmosphere for 16 h. The solution was diluted with EtOAc, filtered through Celite, and washed with water and brine. The organic layer was dried and concentrated. The solid was triturated in ether and filtered to give 86 mg of product (61%). An analytical sample was recrystallized from 2-propanol: mp 149−150 °C; ^{1}H NMR (360 MHz, $CDCl_3$) δ 2.41(s, broad, 3H), 2.62 (s, broad, 4H), 3.83 (s, broad, 2H), 3.95 (s, broad, 2H), 7.68−7.76 (m, 2H), 8.19 (s, 1H), 8.33 (d, J = 7.2 Hz, 1H), 8.37 (d, J = 7.2 Hz, 1H), 8.64 (d, J = 7.2 Hz, 1H), 9.11−9.16 (m, 2H); MS (*m/e*) $[M + H]^+$ 378. Anal. ($C_{20}H_{19}N_5O_3$) C, H, N, O.

Inhibition of Human cAMP-Specific PDE Isoenzymes. PDE3: PDE3 was prepared from human platelets by ultrasonic homogenization. Platelets were washed once with PBS and suspended in 10 mL of buffer (sucrose 0.25 M, EDTA 1 mM, Tris 10 mM, dithiothreitol 1 mM, adjusted to pH 7.4 with HCl) and the following protease inhibitor solutions: 5 mL/mL of phenylmethanesulfonyl fluoride (7 mg/mL in 2-propanol), 1 mL/mL leupeptin and pepstatin A (1 mg/mL each, in ethanol). After sonication (15 s at 4 °C; Branson probe sonicator), homogenates were centrifuged at 2200*g*. The pellet was resuspended in the same volume of buffer H and the sonication repeated. Pooled supernates were stored at −20 °C. Activity was assayed as described.[22]

PDE4: Activity was assessed as previously described.[22] With the exception of PDE4B (rat, expressed in *S. cerevisiae*; human PDE4B is 97% homologous to rat PDE4B), all isoenzyme preparations were from human sources; PDE4A,C,D were expressed in *S. cerevisiae*.

Rolipram Receptor Binding Assay Method. Rat brains were suspended in 10 mL/g of ice-cold buffer (Tris 20 mM, $MgCl_2$ 1 mM, dithiothreitol 0.1 mM, pH 7.5), homogenized using 2 bursts (30 s each) of a Polytron homogenizer (Kinematica, Switzerland), and centrifuged (600*g*, 10 min, 4 °C). The supernatants were pooled, and centrifuged (24000*g*, 10 min, 4 °C). The pellets were resuspended in 5 mL/g of buffer. Protein content was determined using the method of Brad-

ford,[23] according to the instructions of the manufacturer (BioRad). The assay was carried out as described by Schneider et al.[18]

Oxidative Burst from Human Eosinophils. Blood was obtained from normal individuals. Granulocytes were separated from mononuclear cells by Ficoll hypaque gradient centrifugation. Erythrocytes were lysed by two cycles of hypotonic lysis and the remaining granulocytes were incubated with anti-CD16 coated immunomagnetic particles (Miltenyi Biotec, Bergisch Glasbach, Germany). Magnetically labeled neutrophils were then depleted by passing the granulocytes through a MACS (magnetic cell separation) column which resulted in a more than 98% pure eosinophil preparation. These purified human eosinophils were diluted in HBSS and pipetted into 96-well microtiter plates (MTP) at 10^4 cells/well. Each well contained a 200-μL sample comprising: 100 μL of eosinophil suspension, 50 μL of HBSS, 10 μL of lucigenin, 20 μL of activation stimulus, 20 μL of compound of interest. The samples were incubated with compound (dissolved in DMSO and thereafter diluted in buffer) or vehicle for between 10 and 30 min prior to addition of fMLP (0.01−10 μM). MTPs were agitated (Titertek MTP mixer) to facilitate mixing of the cells and medium and then placed into a Hamamatsu luminometer. Total chemiluminescence and the temporal profile of each well was measured simultaneously over 20 min and the results were expressed as a percentage of fMLP-induced chemiluminescence in the absence of compound. Results were fitted to the Hill equation and IC_{50} values calculated. For each compound three independent measurements were done using eosinophils from three different normal donors.

Brown Norway Rat Model of Airway Inflammation. Sensitization, challenge, and bronchoalveolar lavage fluid collection: Male Brown Norway rats (approximately 200 g) were used for the study of cell accumulation. Food and water were available ad libitum. Ovalbumin (OA) (20 mg/mL) was mixed in a blender (Polytron, Kinematica Ltd.) with aluminum hydroxide (20 mg/mL) and injected (sc) concomitantly with *B. pertussis* vaccine (0.25 mL/animal ip) on days 1, 15, and 21. On day 28, sensitized animals were restrained in plastic tubes and exposed (1 h) to an aerosol of OA (3.2 mg/mL) using a nose-only exposure system. Animals were killed 48 h later with pentobarbital (250 mg/kg ip). The lungs were lavaged using 3 aliquots (4 mL) of Hank's solution (100 mL HBSS×10, 100 mL of 0.1 M EDTA solution, 10 mL of 1.0 M HEPES solution, 1 L of water), the recovered cells were pooled, and the total volume of recovered fluid was adjusted to 12 mL by addition of Hank's solution. Compounds were dissolved in DMSO (10 mg in 0.1 mL), diluted with neoral placebo (10 mL), and administered by gavage 1 h prior to and 24 h after antigen exposure. Control groups of actively sensitized animals received saline alone with or without exposure to antigen.

BALF cell counting: Erythrocytes in lavage fluid were lysed (Quicklyser QLA-200A, TOA Medical Electronics Ltd., Japan). Smears were prepared by diluting the recovered fluid (to approximately 10^6 cells/mL) and centrifuging an aliquot. Smears were air-dried, fixed using a solution of fast green in methanol (2 mg/mL) for 5 s, and stained with eosin G (5 s) and thiazin (5 s) in order to differentiate cell types. A total of 500 cells/smear were counted by light microscopy under oil immersion (×1000).

Protein determination: The concentration of protein in BALF supernatants was measured by a colorimetric assay (Bio-Rad DC protein assay). The assay is based upon the reaction of a protein with an alkaline copper tartrate solution and Folin reagent. Briefly, 5 mL of BALF supernatant was added to a 96-well ELISA plate (NUNC). A mixture containing alkaline copper tartrate solution (reagent A′; 25 mL) and dilute Folin reagent (reagent B; 200 mL) was added to each well and incubated for 15 min at room temperature. The absorbance was measured at 700 nm. A bovine serum albumin standard was used to generate a standard curve; the sensitivity of the assay was 0.04 mg/mL protein.

Determination of eosinophil peroxidase (EPO) activity in BALF: The method utilized is based on the oxidation of *o*-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H_2O_2). BALF (10 μL) was mixed with 100 μL of substrate (1 mM OPD, 1 mM H_2O_2, 0.1% Triton-X100, dissolved in 50 mM Tris-HCl, pH 8.0) in a 96-well flat bottom microtiter plate and incubated for 30 min at room temperature. The reaction was stopped by adding 50 μL of H_2SO_4 (4 M) and absorbance was measured at 492 nm in a microplate absorbance spectrophotometer. The concentration of EPO was calculated as units/mL according to the activity of serial dilutions of a standard horseradish peroxidase (Sigma;, 210 U/mg dry wt).

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