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A new chemical tool for exploring the role of the PDE4D isozyme in leukocyte function

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Abstract—Nicotinamide (**2**) is a potent and selective inhibitor of the PDE4D isozyme and as a chemical tool selectively blocks eosinophil mediator release and chemotaxis thus linking the role of PDE4D to eosinophil function.

The phosphodiesterase (PDE) enzyme family controls intercellular levels of secondary messenger cAMP or cGMP through regulation of their hydrolysis. Phosphodiesterase Type IV (PDE4) regulates cAMP through its hydrolysis to 5′-AMP which is blocked by Rolipram (**1**).[1] The PDE4 gene encodes four distinct isoenzyme subtypes designated PDE4A, PDE4B, PDE4C, and PDE4D in which their catalytic sites are conserved but differ in their regulatory domain.[2] Insights into the function of these subtypes have been obtained largely through expression profiling and deficient mice, yet much about the function of these subtypes is unknown or not well understood. The PDE4D subtype is found in most human tissues and organs[2] but is highly expressed in eosinophils,[3] a key leukocyte believed to be involved in the progression of respiratory diseases.[4] In addition, studies in PDE4D deficient mice indicate that PDE4D plays a critical role in airway tone and hyper-reactivity, suggesting a possible further link between PDE4D and respiratory diseases.[5] Archetypical PDE4 inhibitor Rolipram (**1**) has been successfully used as a chemical tool in linking PDE4 with an array of disease states (Fig. 1).[6] However, Rolipram (**1**) shows no significant selectivity in inhibition between the PDE4A, PDE4B, and PDE4D isozymes, and therefore would be ineffective as a chemical tool in defining the physiological function of these isozymes (Table 1). In recent years, a series of naphthyridine PDE4D inhibitors, such as NVP-ABE171, have been described which show efficacy in blocking leukocyte oxidative burst and mediator release in vitro and antigen induced influx and activation in vivo.[7] We have identified nicotinamide derivative **2** as a potent and selective inhibitor of the PDE4D isozyme and as such can be used as a chemical tool in further exploring the role of PDE4D in leukocyte function.

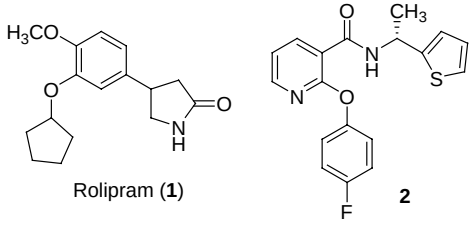

Figure 1. Chemical structures of Rolipram (**1**) and nicotinamide (**2**).

In evaluation of potency and selectivity across the PDE family, both Rolipram (**1**) and (**2**) show selectivity in inhibiting PDE4 over other isozymes (Table 1).[8] However, **2** showed increased potency and selectivity toward PDE4D compared to Rolipram (**1**), which showed no significant differentiation between the PDE4A, PDE4B, and PDE4D isozymes.[9]

Keywords: Phosphodiesterase; PDE4D; Eosinophil.
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Table 1. Potency and selectivity of Rolipram (**1**) and nicotinamide (**2**)

PDE isozyme	Rolipram (**1**)	Nicotinamide (**2**)
PDE2 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE3 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE4A IC_{50}, nM (*n*)[a]	11 (8)	107 (6)
PDE4B IC_{50}, nM (*n*)[a]	34 (8)	185 (6)
PDE4C IC_{50}, nM (*n*)[a]	3410 (2)	1378 (2)
PDE4D IC_{50}, nM (*n*)[a]	33 (8)	4 (6)
PDE5 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE8A IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE8B IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE9 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE10 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE11 IC_{50}, nM (*n*)	>16,000 (1)	>16,000 (1)
PDE4D selectivity	<1X	27X

[a] Values are means of a number (*n*) of experiments.

In considering that **2** might interact with other pharmacological targets that in turn could then compromise interpretation of results from further in vitro and in vivo functional profiling, **2** was assayed for interaction against a collection of 52 receptors and ion channels where no significant level of binding relative to its PDE4D potency was found.[10]

Having established selectivity in **2** for PDE4D over other potential pharmacological targets, we next wanted to profile it for functional activity. Inhibition of PDE4 elevates intracellular levels of cAMP by inhibiting hydrolysis to 5′-AMP and PDE4D has been found to be expressed in U937 cells.[2,11] In these cells, **2** was found to be 50-fold more potent in elevating intracellular cAMP compared to Rolipram (**1**) (Table 2). The elevation of intracellular cAMP has been shown to block leukocyte mediator release.[12] With this in mind and considering that PDE4D may be linked to eosinophil function, we wished to evaluate **2** against several cellular functional endpoints. Degranulation and respiratory burst of eosinophils result in the release of the intercellular mediators eosinophil derived neurotoxin (EDN) and cysteinyl leukotrienes (LTE_4), which are associated with airway inflammation and constriction responses, respectively.[13] In blocking the release of EDN and LTE_4 in human whole blood, **2** proved to be approximately 20-fold more potent than Rolipram (**1**).[14] Tumor necrosis factor (TNF-α) is another inflammatory mediator that is regulated by intercellular cAMP and is produced primarily by monocytes.[15] Interestingly, **2** had little effect on blocking the release of TNF-α in human whole blood relative to those on EDN and LTE_4. This effect is likely due to **2** selectively inhibiting PDE4D, the predominant isozyme in eosinophils, and consequently being less potent than Rolipram (**1**) in inhibiting PDE4B, the predominant isozyme in monocytes which has been shown to regulate TNF-α production.[16]

Leukocyte chemotaxis is a key step in the inflammatory response and PDE4 inhibitors are known to inhibit leukocyte chemotaxis by elevating intracellular cAMP.[17] With this in mind and considering that PDE4D may be linked to eosinophil function, we wished to examine the effect of **2** on in vitro human eosinophil chemotaxis.[18] Against chemoattractant mediators leukotriene B_4 (LTB_4), C5a, platelet-activating factor (PAF), and eotaxin, **2** proved to be several orders of magnitude more potent than Rolipram (**1**) in blocking eosinophil chemotaxis (Table 3). Having demonstrated that **2** was effective in inhibiting eosinophil chemotaxis in vitro, we next wished to examine the effect of **2** on leukocyte chemotaxis in vivo. In regard to in vivo evaluation of nonselective PDE4 inhibitors, there has been increasing evidence suggesting that the occurrence of emesis in nonselective PDE4 inhibitors like Rolipram (**1**) may be due to inhibition of the PDE4D subtype.[19] It was found that **2** possesses suitable physiochemical and ADME properties for aerosol delivery directly into the airways, which would allow for evaluation of effects on leukocyte chemotaxis in vivo while minimizing potential emetic side effects due to systemic exposure.[20] Rolipram (**1**) has been shown to be efficacious in reducing the influx of neutrophils and eosinophils in bronchoalveolar lavage (BAL) fluid from cynomolgous monkeys exposed to *Ascaris suum*.[21] In this model, aerosol delivery of **2** significantly reduced the infiltration of eosinophils (% inhibition = 65 ± 14, $p < 0.05$) but had no significant effect on neutrophil influx.[22] The selectivity observed in reducing in vivo eosinophil over neutrophil influx is likely due to **2** selectively inhibiting PDE4D, the predominant isozyme in eosinophils, and consequently being less potent than Rolipram (**1**) in inhibiting PDE4B, a predominant isozyme in neutrophils.[23]

The synthesis of **2** is carried out using a linear three-step sequence and a resolution by chiral HPLC (Scheme 1).

Table 3. Comparative effect of Rolipram (**1**) and nicotinamide (**2**) on in vitro human eosinophil chemotaxis

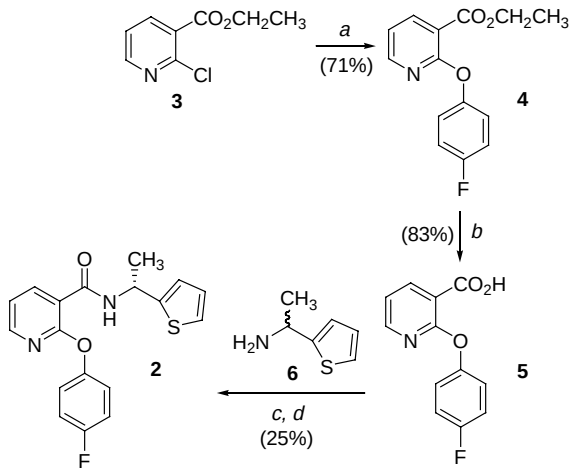
Chemoattractant	Rolipram (**1**)	Nicotinamide (**2**)
LTB_4 IC_{50}, nM (*n*)[a]	6500 (2)	<1 (2)
C5a IC_{50}, nM (*n*)[a]	17,000 (2)	1 (2)
PAF IC_{50}, nM (*n*)[a]	3000 (2)	1 (2)
Eotaxin IC_{50}, nM (*n*)[a]	13,000 (2)	3 (2)

[a] Values are means of a number (*n*) of experiments.

Table 2. Comparative in vitro functional activity of Rolipram (**1**) and nicotinamide (**2**)

Functional assay	Rolipram (**1**)	Nicotinamide (**2**)
cAMP elevation U937 cells EC_{50}, nM (*n*)[a]	1100 (16)	22 (2)
EDN human whole blood IC_{30}, nM (*n*)[a]	283 (5)	16 (4)
LTE_4 human whole blood IC_{50}, nM (*n*)[a]	41 (3)	2 (4)
TNF-α human whole blood IC_{50}, nM (*n*)[a]	625 (2)	925 (3)

[a] Values are means of a number (*n*) of experiments.



Scheme 1. Reagents and conditions: (a) 4-fluorophenol, Cs_2CO_3, DMF, 80 °C, 18 h; (b) 1 N NaOH, EtOH, reflux, 3 h; (c) (i) $ClCO_2CH_2CH(CH_3)_2$, *N*-methylmorpholine, CH_2Cl_2, −10 °C, 20 min; (ii) **6**, −10 °C to 20 °C, 18 h; (d) Chiral HPLC, Chiralcel AS column, EtOH/heptane (1:9) elutant.

Displacement of the activated 2-chloro substituent in **3** with 4-fluorophenol in the presence of cesium carbonate in dimethylformamide at 80 °C gives **4** which in turn is saponified with aqueous sodium hydroxide in refluxing ethanol to give carboxylic acid **5**.[24] Coupling of the mixed isobutyl anhydride of **5** with **6** followed by resolution and isolation of the dextrorotatory enantiomer by chiral HPLC affords **2** in 99% enantiomeric purity which is assigned the *R*-configuration based on literature analogy.[25]

Nicotinamide (**2**) is a potent and selective inhibitor of the PDE4D subtype over the PDE4A, PDE4B, and PDE4C subtypes and shows no inhibition of other PDE isozymes. In addition, when evaluated against a collection of 52 receptors and ion channels, **2** shows no significant binding. In cellular function, by selectively inhibiting PDE4D, **2** effectively elevates intracellular cAMP and blocks the release of eosinophil associated mediators EDN and LTE_4 in human whole blood. However, **2** is ineffective in blocking the release of monocyte associated mediator TNF-α from human whole blood compared to Rolipram (**1**), presumably due to the predominant subtype in monocytes being PDE4B, which has been shown to selectively regulate TNF-α. Nicotinamide (**2**) is effective in blocking chemokine stimulated eosinophil chemotaxis in vitro and in vivo selectively reduces the influx of eosinophils over neutrophils in bronchoalveolar lavage (BAL) fluid from cynomolgous monkeys exposed to *Ascaris suum*, presumably due to PDE4D being the predominant PDE4 subtype in eosinophils over neutrophils. These findings demonstrate that PDE4D inhibitor **2** is an effective chemical tool implicating PDE4D in playing a unique role in eosinophil chemotaxis and mediator release.

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