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Phosphodiesterase 4 Inhibitors as Airways Smooth Muscle Relaxant Agents: Synthesis and Biological Activities of Triazine Derivatives

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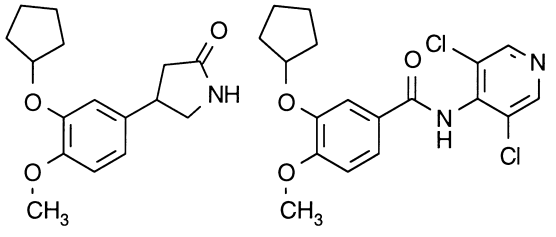
Abstract—A series of triazine derivatives was synthesized. The compounds were evaluated for tracheal smooth muscle relaxant and type 4 phosphodiesterase inhibitory activities. A highly significant correlation was observed between the two effects. Two compounds exhibited potent relaxant activity (EC_{50}: 17 and 24 nM) and might be useful for the treatment of asthma.

Introduction

Cyclic nucleotide phosphodiesterases (PDE) control the rate of breakdown of the intracellular messengers, 3′,5′-cyclic adenosine and guanosine monophosphates (cAMP, cGMP), by hydrolyzing the 3′-ribose phosphate bond of the nucleotides to form 5′-nucleoside monophosphates. Currently, more than 30 isoenzymes are recognized and grouped into at least seven different families, each with distinct substrate specificities and regulatory characteristics.[1–6] The concept of differently expressed and regulated PDE isoenzymes implies that individual PDEs are likely to be good targets for therapeutic intervention in diseases caused or regulated by cyclic nucleotide-modulated transduction mechanisms.

Asthma is an inflammatory condition characterized by bronchoconstriction, microvascular leakage, mucus hypersecretion, bronchial hyperresponsiveness and by invasion of the airways by inflammatory cells.[7,8] The PDE 4 family has a marked preference for cAMP as a substrate and, as a consequence, inhibition of PDE 4 activity results in increased intracellular levels of this nucleotide, which is generally associated with dampening effects on airway smooth muscle tone and on activation and mediator release from inflammatory cells. Thus, on the basis of the presence of the type 4 PDE in airway smooth muscle[9–12] and in proinflammatory cells,[13–17] and of the effects of the PDE 4 inhibitors such as rolipram or RP 73401 in experimental studies,[3,4,18–23] it is generally accepted that inhibitors of this isoenzyme may have useful bronchodilator and anti-inflammatory properties for treating both the symptoms and the underlying causes of the disease.



Rolipram RP 73401

This study focuses on the synthesis and pharmacological evaluation of triazine derivatives, designed by structural analogy with cAMP. We present the guinea pig tracheal relaxant effect of these compounds, which was found to be highly correlated with their ability to inhibit PDE 4 catalytic activity. Two compounds exhibit potent relaxant activity, comparable to the activity displayed by RP 73401.

Chemistry

The *s*-triazine derivatives reported in this paper are listed in Table 1. In general, these derivatives and intermediates were prepared by the methods illustrated in Scheme 1 and Scheme 2. 2-(Cyclo)alkyl-4,6-dichloro-*s*-triazines (**II**) were synthesized by the reaction of 2,4,6-trichloro-*s*-triazine (**I**) with Grignard reagents according

Key words: Bronchodilators; enzyme inhibitors; substituent effects; antiinflammatories.
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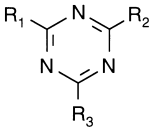
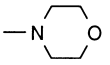
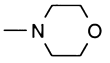
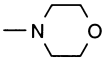
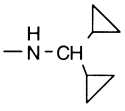
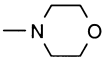
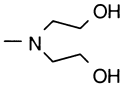
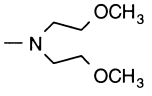
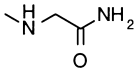
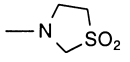
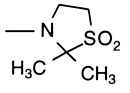
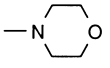
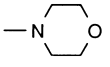
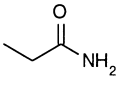
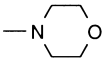
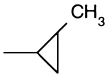
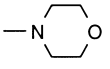
Table 1. Comparative effects of triazine derivatives, rolipram, and RP73401 on guinea pig tracheal smooth muscle and on polymorphonuclear type 4 PDE activity



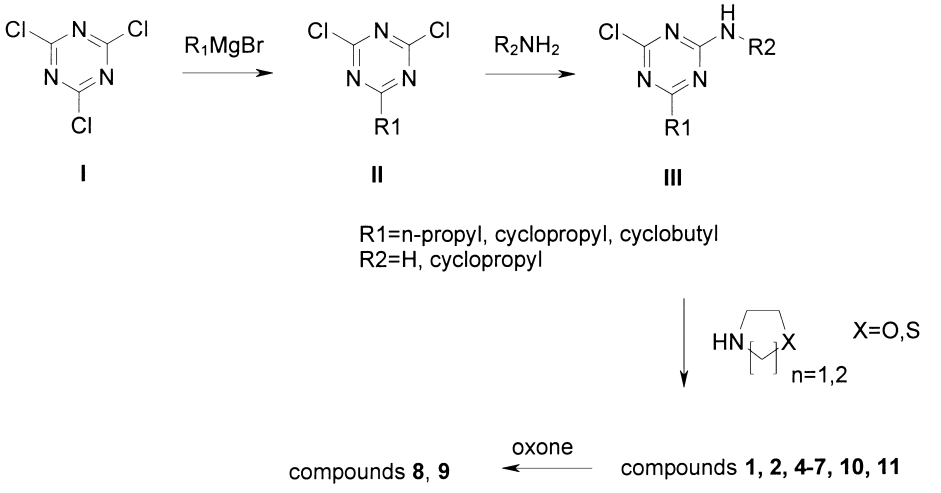
				Tracheal relaxation		PDE 4 inhibition
	R_1	R_2	R_3	EC_{50} (μM)	1 μM-induced effect[a]	IC_{50}(μM)
1				0.052[b] ± 0.029	58 ± 9	0.16 ± 0.11(3)
2				0.49 ± 0.30	67 ± 15	5.68
3				0.71 ± 0.22	63 ± 7	2.94
4				1.5 ± 0.2	42 ± 3	24.5
5				0.15 ± 0.06	83 ± 8	0.69
6				0.47 ± 0.11	69 ± 10	5.50
7				2.5 ± 1.4	39 ± 13	30.1 ± 12.8(2)
8				0.017[b] ± 0.002	77 ± 7	0.14 ± 0.10(4)
9				1.7 ± 0.9	50 ± 23	53.3
10				0.076[b] ± 0.027	45 ± 2	0.38 ± 0.39(4)
11				0.12[b] ± 0.07	61 ± 15	0.33 ± 0.19(3)
12				90 ± 3.3	20 ± 3	61.2 ± 16.0(3)
13				0.021[b] ± 0.023	59 ± 4	0.18 ± 0.04(2)
14(d)				0.024[b] ± 0.011	78 ± 10	0.15 ± 0.01(6)
15				0.16 ± 0.02	77 ± 2	1.96 ± 0.33(3)
Rolipram				0.014[b] ± 0.008	40 ± 11	0.09 ± 0.03(8)
RP73401				0.010 ± 0.006	76 ± 7	0.004 ± 0.001(2)

Relaxation values are the means of three determinations; the number of PDE 4 inhibitory experiments is indicated in brackets; [a]% of maximal relaxation to isoprenaline (1 mM); [b]EC_{50} values for the first relaxant effect below 1 μM (agents giving biphasic curves, see text); (d) dextrogyre.

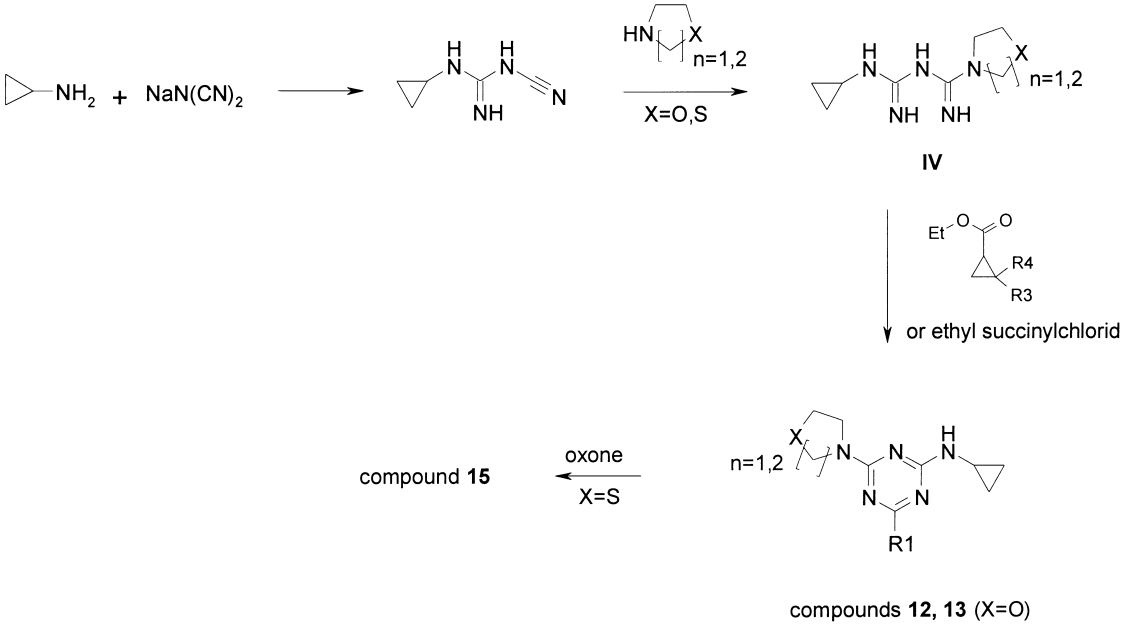
to Hirt et al.[24] The intermediates **III** were obtained by treatment of **II** with an equimolar amount of the appropriate amine in the presence of K_2CO_3, according to the method of Nishigaki et al.[25] Compounds **1**, **2**, **4**–**7**, **10**, and **11** were obtained from the reaction of **III** with two equivalents of the appropriate amine or one equivalent of amine in the presence of triethylamine in dioxane at reflux temperatures.[26] Substituted cycloalkyl-*s*-triazines **13** and **14** were prepared from the corresponding biguanides (**IV**) and the appropriate substituted cycloalkyl carboxylic esters or carboxylic acid chloride[27–30] (Scheme 2). Compound **12** was obtained by this procedure using ethyl succinyl chloride and subsequent aminolysis of the intermediary ester. *S*,*S*-dioxides **8**, **9**, **14**, and **15** were obtained by oxidation with oxone[31] of the corresponding sulfide derivatives, prepared according to either of the procedures described.

Results and Discussion

Triazine derivatives relaxed the guinea pig tracheal smooth muscle with varying potencies and efficacies (testing concentrations from 1 nM to 100 μM). Two groups were discerned. In the first group (**1**, **8**, **10**, **11**, **13**, **14**; EC_{50} from 17 to 120 nM, Table 1), compounds relaxed isolated trachea in a biphasic manner as demonstrated by the differences in curve slope at low, middle and high concentrations (Fig. 1). They were more potent than the compounds of the second group whose concentration–relaxation relationships appeared to follow sigmoidal curves (**2**–**7**, **9**, **12**, **15**; EC_{50} from 150 nM to 9 μM). The first phase was observed for concentrations below 1 μM. Depending on the compound tested, this first phase ended in a marked plateau or an inflexion of the curve, between 46 and 73% of the maximal



Scheme 1.



Scheme 2.

relaxation achieved with 1 mM isoprenaline. The second phase occurred with upper concentrations (> 1 μM) and gave nearly the maximal smooth muscle relaxation in most cases.

Among the triazine derivatives giving biphasic curves, two different types could be discerned: the rolipram type and the RP73401 type. In the case of rolipram (Fig. 1A), the concentration–response curve was markedly biphasic and reached only 37% of the maximal isoprenaline-induced tracheal relaxation during the first phase. On the other hand, RP 73401 mean curve was sigmoidal (Fig. 1B) and reached 76% of the maximal isoprenaline-induced tracheal relaxation at 1 μM. This relaxation was not significantly improved with higher RP73401 concentrations (80% at 100 μM). Despite differences between the profile of their relaxation curve, the EC_{50} values for rolipram and RP73401 relaxant effect below 1 μM were nearly identical: 14 and 10 nM respectively. The relaxant effects elicited by these specific PDE 4 inhibitors, used as reference products, were consistent with the results provided in the literature.[19,32]

The concentration–relaxation curves for compounds **8** and **14** resembled the curve obtained for RP73401 (Fig. 1B) with an additional effect above 1 μM. On the contrary, the concentration–response curve for **10** was rather similar to rolipram (Fig. 1A), but shifted to higher concentrations. The derivatives **1**, **11**, and **13** gave an intermediary response (Fig. 1A). As a consequence of the intermediate plateau in their relaxation curve, rolipram and the triazine derivatives **1**, **10**, **11**, **13**, displayed a smaller absolute effect at 1 μM than some compounds giving a sigmoidal curve (Table 1).

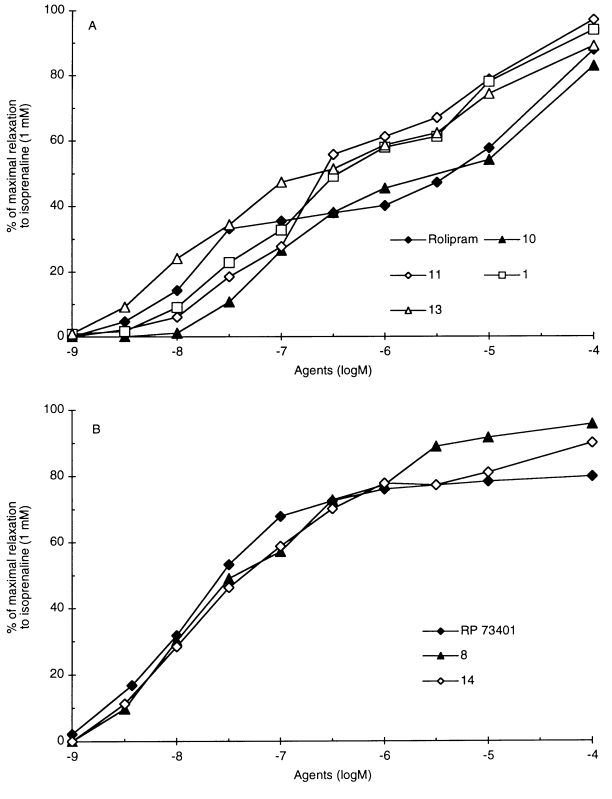


Figure 1. Biphasic cumulative concentration–relaxation curves of rolipram and compounds **1**, **10**, **11**, and **13** (A) and RP73401 and compounds **8** and **14** (B). For clarity, SE have not been reported.

The biphasic nature of some concentration–relaxation relationships suggested that there might be at least two mechanisms at the origin of the triazine derivatives mediated-relaxation of guinea pig trachea. We examined the inhibitory activity of these compounds on type 4 phosphodiesterase isoenzymes from polymorphonuclear leukocytes and determined IC_{50} values ranging from 0.14 to 61.2 μM (Table 1). Moreover, we observed a highly significant correlation ($R^2 = 0.93$, $n = 15$, Student: $p > 0.1\%$, Fig. 2) between the ability of triazine derivatives to inhibit PDE 4 isoenzymes and their ability to relax trachea (using EC_{50} value for the first relaxant effect occurring below 1 μM in the case of biphasic curves). Thus, one part of the triazine-relaxant effect, and in particular the first effect that occurred with biphasic curves giving compounds used at concentrations below 1 μM, may be due to PDE 4 isoenzyme inhibition. For these last compounds (**1**, **8**, **10**, **11**, **13**, **14**), complete relaxation may be achieved when another mechanism is associated. For instance, A. L. Harris and his collaborators[32] observed that pretreatment of the trachea with a fixed concentration of CI-930, a selective inhibitor of PDE 3 isozyme, converted the rolipram biphasic curve into a sigmoidal curve, near complete below 1 μM. Thus, in the case of rolipram, the first relaxant effect may be due to the specific PDE 4 isoenzyme inhibition, as very elevated concentrations of agent may inhibit PDE 4 and PDE 3 isoenzymes.

In summary, these results show that compounds **8** and **14** may be the most interesting agents in this series of triazine derived PDE 4 isoenzyme inhibitors because they combine a low EC_{50} (respectively 17 and 24 nM) with an important effect during the first phase of the tracheal relaxation (73 and 64% of maximal relaxation to isoprenaline). These [1,3,5]triazine derivatives are ideally substituted with a cyclopropylamino group into the 2 position (substituent R_1, Table 1), with a cyclopropyl or

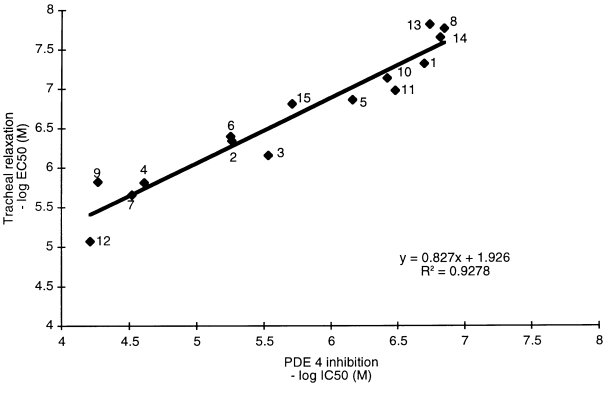


Figure 2. Guinea pig tracheal relaxation as a function of polymorphonuclear PDE 4 inhibition. EC_{50} values (expressed in ordinate) are either the concentrations of relaxant agent that produced 50% of the maximal effect, or the concentrations that produced 50% of the first relaxant phase, according to the sigmoidal or biphasic aspect of the curves. Abscissa: inhibition of polymorphonuclear PDE 4.

a 2-methyl-cyclopropyl group into the 4 position (R_2) and with a 1,1-dioxo-1,3-thiazolidine or a 1,1-dioxo-1,4-thiomorpholine ring in the 6 position (R_3).

The replacement of the pre-cited substituents with a propyl or a cyclobutyl group into the 4 position (R_2) or with a morpholine ring into the 6 position (R_3) lead to compounds (**1**, **10**, **11**, **13**) slightly less potent than **8** and **14** (according to their EC_{50} values), but less efficacy during their first relaxant phase (according to the percentage of maximal relaxation achieved). All the other modifications listed in Table 1 are associated with a great decrease on the relaxant and PDE 4 inhibition parameters.

Since the triazine derivatives **8** and **14** are more selective inhibitors of the type 4 PDE (**8** and **14** platelet PDE 3 inhibitory activity[33]: $IC_{50} > 100\,\mu M$, versus 0.14 and 0.15 μM for the PDE 4 inhibitory activity), our results confirm that the PDE 4 isoenzyme is functionally important in modulating the tracheal smooth muscle tone in the guinea pig. It should be noted that selective inhibitors of the type 3 PDE relax guinea pig trachea in vitro.[4,32] However, PDE 3 inhibitors have been shown to induce positive inotropism and vasodilation whereas inhibition of type 4 PDE is not associated directly with such cardiovascular effects.[3] Thus, type 4 PDE inhibitors as **8** and **14** may be more selective and useful for the treatment of asthma.

Experimental

Chemistry

^{1}H NMR spectra were recorded on a Bruker AM-250 spectrometer unless otherwise stated. Low-resolution mass spectra were recorded with a Kratos or a Finnigan TSQ 700 mass spectrometer. Normal-phase silica gel (Merck, silica gel 60 F_{254}) was used for chromatography. Products were purified by preparative HPLC over silica gel (Merck, silica gel 60, size 0.014–0.040 mm).

Compounds **1**, **5**, **8**, **10**, **11**, **13**, **14**, and **15** have been described previously.[26]

4-Cyclopropyl-6-morpholin-4-yl-[1,3,5]triazin-2-yl-amine (2). 2.69 g (15.7 mmol) of 2-amino-4-chloro-6-cyclopropyl-[1,3,5]triazine (prepared from 2,4-dichloro-6-cyclopropyl-[1,3,5]triazine and aqueous ammonia in acetone[25]) and 3 g (35 mmol) of morpholine in 50 mL of dioxane were heated at 100 °C for 2 h. The mixture was cooled to room temperature, concentrated under vacuum and partitioned between 50 mL of dichloromethane and 50 mL of water. The layers were separated and the organic phase was dried over sodium sulfate, filtered and concentrated. The residue was purified over silica gel, eluent dichloromethane:ethanol (97.5:2.5, v/v). The product **2** was isolated as the hydrochloride from ethanol and diethylether: Yield 3.25 g (80.4%); mp 203–204 °C; ^{1}H NMR (DMSO-d_6) δ 1.22 (m, 4H), 2.00 (m, 1H), 3.64 (m, 4H), 3.77 (m, 4H), 7.5–8.7 (broad, 3H); MS M^+ 221; Anal. ($C_{10}H_{15}N_5O$·HCl).

Cyclopropyl-(4-cyclopropyl-6-morpholin-4-yl-[1,3,5]triazin-2-yl)-methyl-amine (3). 3.5 g (11 mmol) of compound **12** was dissolved in 35 mL of dimethylformamide and 0.76 g (25 mmol) of 80% NaH in mineral oil were added. The mixture was heated at 50 °C for 30 min. After cooling to room temperature 1.66 g (1.1 mL, 40 mmol) of methyl iodide was added. The mixture was heated at 50 °C for 1 h, cooled and concentrated. The residue was partitioned between 50 mL of dichloromethane and 50 mL of water and the layers were separated. The organic phase was dried over sodium sulfate, filtered, and concentrated. The residue was purified over silica gel, eluent dichloromethane:ethanol (49:1, v/v). The product was recrystallized as the hydrochloride from a mixture of methanol and diethylether giving 2.9 g of **3**: Yield 80%; mp 109–110 °C; ^{1}H NMR (DMSO-d_6) δ 0.79 (m, 2H), 0.93 (m, 2H), 1.15 (d, 3H), 2.9 (m, 1H), 3.65 (m, 4H), 3.80 (m, 4H); MS M^+ 275; Anal. ($C_{14}H_{21}N_5O$ ·HCl).

(4-Cyclopropyl-6-morpholin-4-yl-[1,3,5]triazin-2-yl)-dicyclopropylmethyl-amine (4). 2.4 g (10 mmol) of 2-chloro-4-cyclopropyl-6-morpholin-4-yl-[1,3,5]triazine,[26] 1.1 g (10 mmol) of dicyclopropylmethylamine (prepared from dicyclopropylketone and hydroxylamine and subsequent reduction of the imine with sodium metal in ethanol)[34] and 1.5 mL of triethylamine in 100 mL of dioxane were heated at reflux (100 °C) for 3 h. The mixture was cooled to room temperature and concentrated under vacuum. The residue was partitioned between 50 mL of dichloromethane and 50 mL of water. The layers were separated and the organic phase was dried over sodium sulfate, filtered, and concentrated. The residue was purified over silica gel, eluent ethylacetate-*n*-hexane (1:9, v/v). The product was recrystallized as the hydrochloride from a mixture of methanol and ethyl acetate giving 1.3 g of **4**: Yield 37%; ^{1}H NMR (DMSO-d_6) δ 0.3–0.6 (m, 8H), 1.00–1.3 (m, 6H), 1.95 (m, 1H), 3.21 (m, 1H), 3.6 (m, 4H), 3.77 (m, 4H), 8.41 (d, 1H); MS M^+ 315; Anal. ($C_{17}H_{25}N_5O$·HCl).

6,*N*-Dicyclopropyl-*N*′,*N*′-bis(2-methoxyethyl)-[1,3,5]triazine-2,4-diamine (6). 6.3 g (0.03 mol) of 2-chloro-4-cyclopropyl-6-cyclopropylamino-[1,3,5]triazine[26] and 8 g (0.06 mol) of bis(2-methoxyethyl)-amine in 100 mL of dioxane were heated at 100 °C for 2 h. The brown mixture was cooled to room temperature, concentrated under vacuum, and taken up in 100 mL of dichloromethane. After washing with water (2×100 mL) and drying over sodium sulfate, the solution was concentrated leaving 10 g of product that was purified over silica gel using eluent dichloromethane:ethanol (49:1, v/v). After crystallization from *n*-hexane there were obtained 5.5 g of **6**: Yield 59.7%; mp 64–66 °C; ^{1}H NMR ($CDCl_3$) δ 0.50 (m, 2H), 0.73 (m, 2H), 0.84 (m, 2H), 1.05 (m, 2H), 1.76 (m, 1H), 2.74 (m, 1H), 3.35 (s, 6H), 3.56 (m, 4H), 3.76 (m, 4H), 5.13 (s, 1H); MS M^+ 307; Anal. ($C_{15}H_{25}N_5O_2$).

2-(4-Cyclopropyl-6-cyclopropylamino-[1,3,5]triazin-2-yl-amine)-acetamide (7). 6.3 g (0.03 mol) of 2-chloro-4-cyclopropyl-6-cyclopropylamino-[1,3,5]triazine,[26] 4.42 g (0.04 mol) of glycinamide hydrochloride, and 11 g

(0.08 mol) of potassium carbonate in 70 mL of isopropanol were heated at reflux (80 °C) for 50 h. The mixture was cooled to room temperature and concentrated under vacuum. The residue was partitioned between 200 mL of dichloromethane and 200 mL of water. The solids were removed by filtration and the layers were separated. The organic phase was dried over sodium sulfate, filtered, and concentrated. The residue was purified over silica gel using eluent dichloromethane: ethanol (9:1, v/v). The product was recrystallized from a mixture of ethyl acetate and *n*-hexane giving 1.86 g of **7**: Yield 25%; mp 187–188 °C; ^{1}H NMR (DMSO-d_6) δ 0.46 (m, 2H), 0.62 (m, 2H), 0.84 (m, 2H), 0.93 (m, 2H), 1.68 (m, 1H), 2.72 (m, 1H), 3.76 (m, 2H), 6.88 (broad s, 2H), 7.14 (broad s, 2H); MS M^+ 248; Anal. ($C_{11}H_{16}N_6O$).

Cyclopropyl-[4-cyclopropyl-6-(2,2-dimethyl-1,1-dioxo-1λ^6-thiazolidin-3-yl)-[1,3,5]triazin-2-yl]-amine (9). 22.4 g (146 mmol) of 2,2-dimethylthiazolidine hydrochloride (prepared from 2-aminoethanethiol and acetone) were dissolved in 50 mL of water and 4 N NaOH was added until the pH of the solution reached between 8 and 9 and the free base was extracted with dichloromethane (3×200 mL). The combined organic phases were dried over magnesium sulfate, filtered, and concentrated, giving an oily residue of 15.5 g (132 mmol) of 2,2-dimethylthiazolidine. This residue was dissolved in 200 mL of dioxane and 13.94 g (66.2 mmol) of 2-chloro-4-cyclopropyl-6-cyclopropylamino-[1,3,5]triazine[26] were added. The mixture was refluxed for 35 h, cooled to room temperature, and concentrated under vacuum. The residue was partitioned between 200 mL of dichloromethane and 200 mL of water. The layers were separated and the organic phase was dried over magnesium sulfate, filtered, and concentrated. The yellow residue (24 g) was chromatographed on silica gel, eluent dichloromethane: methanol (99:1, v/v). The product was recrystallized from a mixture of dichloromethane and *n*-hexane giving 7.2 g of the thiazolidine precursor of **9** with a yield of 37.4%. This material was used as such in the next step.

6.6 g (22.7 mmol) of the above obtained material, dissolved in 300 mL of dichloromethane:methanol (1:2, v/v), was oxidized by dropwise addition during 15 min of 27.9 g (45.4 mmol) of oxone ($2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$) in 100 mL of water at 15 °C. The mixture was stirred for 24 h at room temperature. Water (1 L) was added and the mixture was extracted with dichloromethane (3×1 L). The combined washings were dried over magnesium sulfate, filtered, and concentrated. The residue (7 g) was chromatographed on silica gel using eluent dichloromethane:methanol (99:1, v/v). The product was recrystallized from a mixture of ethylacetate and *n*-hexane giving 4.65 g of **9**: Yield 63.5%; mp 164–165 °C; ^{1}H NMR (DMSO-d_6) δ 0.53 (m, 2H), 0.76 (m, 2H), 0.91 (m, 2H), 1.08 (m, 2H), 1.74–1.85 (m + s, 7H), 2.72 (m, 1H), 3.20 (t, 2H, J = 7 Hz), 4.04 (t, 2H, J = 7 Hz), 5.31 (s, 1H); MS M^+ 323; Anal. ($C_{14}H_{21}N_5O_2S$).

3-(4-Cyclopropylamino-6-morpholin-4-yl-[1,3,5]triazin-2-yl-propionamide (12). To a solution of 82 g (0.33 mol) of *N*-[imino(cyclopropylamino)methyl]-4-morpholinecarboximidamide hydrochloride[26] in 1 L of methanol (distilled from sodium) was added a solution of 15.2 g (0.66 mol) of sodium in 500 mL of methanol. The mixture was stirred for 30 min at room temperature, 51.3 mL (0.36 mol) of ethyl succinylchloride was added dropwise with stirring over 30 min while maintaining the temperature of the mixture between 25–35 °C. The mixture was heated at reflux overnight. After cooling and filtering, the mixture was concentrated to dryness, leaving a syrup. The product was purified over silica gel using eluent dichloromethane:methanol (49:1, v/v) yielding 18.5 g (18.2%) of the ester as an oil that crystallized upon standing. 10.2 g (0.033 mol) of the product was dissolved in 1 L of methanol, the mixture was cooled to −15 °C and ammonia gas was bubbled through the solution for 5 days. The mixture was concentrated and the residue was purified over silica gel, eluent dichloromethane:methanol (9:1, v/v) and crystallization from a mixture of methanol and diethylether gave 5.9 g of **12** as a solid: Yield 61.2%; mp 217–218 °C; ^{1}H NMR (DMSO-d_6) δ 0.47 (m, 2H), 0.64 (m, 2H), 2.43 (m, 2H), 2.61 (m, 2H), 2.75 (m, 1H), 3.60 (m, 4H), 3.71 (m, 4H), 6.64 (s, 1H), 7.22 (s, 1H), 7.36 (s, 1H); MS M^+ 292; Anal. ($C_{13}H_{20}N_6O_2$).

Pharmacology

Materials. Guinea pigs and rats were obtained from Charles River (Saint Aubin les Elbeuf, France) and Iffa Credo (Brussels, Belgium), respectively. Isoprenaline and the nucleotidase from snake venom (Crotalus Atrox V-7 000) were obtained from Sigma (Saint-Quentin-Fallavier, France). Hank's balanced salt solution, [2,8-^{3}H]cAMP, AG1-X2 anion exchange resin and Ultimagold-RX were purchased, respectively, from Gibco-Life Technologies (Merelbeke, Belgium), NEN Dupont (Brussels, Belgium), BioRad (Nazareth Eke, Belgium), and Canberra-Packard (Zellik, Belgium). Rolipram and RP73401 were synthesized by UCB Pharma Sector (Braine-l'Alleud, Belgium). All other reagents were of the highest available grade and purchased from standard sources.

Tracheal relaxant activity. Tracheal relaxant activity was measured as previously described with slight modifications.[35] Male albino guinea pigs weighing 350–450 g were anesthetized by an intraperitoneal injection of pentobarbital (50 mg kg^{-1}). The trachea was removed, trimmed of excess tissue, and opened longitudinally by cutting through the cartilage. Six transverse segments, each containing 3 to 4 cartilaginous rings, were prepared. Each strip was suspended in a 15 mL tissue bath and attached via a bristle thread to a force displacement transducer connected to a polygraph for isometric recording of tension. The smooth muscle was bathed in modified Krebs–Henseleit solution of the following composition: NaCl, 120 mM; KCl, 4.75 mM; $CaCl_2$, 2.5 mM; $MgSO_4{\cdot}7H_2O$, 1.2 mM; KH_2PO_4, 1.15 mM; $NaHCO_3$, 25 mM; glucose, 10 mM. Tissue baths were maintained at 37 °C and constantly bubbled with air: 5% CO_2 mixture. The tissues were allowed to equilibrate for 90 min with frequent washings under a resting tension of 1.5 g, which was found optimal for determining changes in tension. Concentration-response curves were constructed by

successive increases in the bath concentration of agent. At the end of each experiment, a supramaximal concentration of isoprenaline (1 mM) was administered and relaxant responses were expressed as a percentage of the maximal isoprenaline relaxation to minimize variability between tissues. Only one cumulative response curve was obtained on a single tissue to avoid problems of previous treatment. EC_{50} values were calculated by linear regression of concentration-response curves generated by concentrations that caused significant effect between 10–90%. Rolipram, RP73401 and triazine derivatives were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide used in each experiment was below 0.2% at which the solvent did not influence assays. Isoprenaline was dissolved in deionized water.

Inhibition of PDE 4 activity. Polymorphonuclear leukocytes were isolated from pleural cavity of rats locally injected with autolog serum, as described by J. P. Giroud and his collaborators with slight modifications.[36] Briefly the cells were collected three hours after serum injection, washed with Hank's balanced salt solution, made free from erythrocytes by hypotonic shock and finally suspended in the following buffer: Tris-HCl pH 7.4, 6.7 mM; EGTA, 1.7 mM; $MgCl_2$, 0.7 mM; $CaCl_2$, 0.6 mM; KCl, 1.8 mM; NaCl, 91 mM; KH_2PO_4, 0.98 mM; NaH_2PO_4, 5.4 mM; glucose, 3.7 mM; gelatine, 0.67 g/L; phenylmethylsulfonyl fluoride, 0.33 mM; dithiothreitol, 0.67 mM. Cells were disrupted by sonication and centrifuged (15 000 *g*, 30 min, 4 °C). Supernatant was used to measure phosphodiesterase activity. Aliquots were frozen in liquid nitrogen and preserved at −70 °C. PDE 4 activity was measured according to Kono[37] and Ong and Rennie[38] with slight modifications. The incubations were made 20 min at 37 °C in the following buffer: dextrose, 0.3 mM; $CaCl_2$, 0.05 mM; KCl, 0.14 mM; KH_2PO_4, 0.08 mM; Na_2HPO_4, 0.43 mM; NaCl, 7.3 mM; Tris, 46.5 mM; $MgCl_2$, 9.55 mM; EGTA, 0.13 mM; dithiothreitol, 4.65 mM; sodium acetate, 4.9 mM. cAMP was at 1 μM (with 200 000 dpm [^{3}H]cAMP) and dimethyl sulfoxide 0.1% v/v. The reaction was stopped by addition of HCl. After enzyme denaturation by heat and neutralization to pH 7.5 with NaOH, the entire [^{3}H]5'cAMP produced was converted into [^{3}H]adenosine by incubation with 5′nucleotidase from snake venom and [^{3}H]adenosine was separated from unreacted [^{3}H]cAMP by column chromatography on AG1-X2 anion exchange resin (0.8 mL) eluted with water. Radioactivity was quantified in a liquid scintillation counter after addition of Ultimagold-XR. Percentage of hydrolyzed cAMP must be included between 6–25%. Inhibition by 0.1 μM rolipram tested simultaneously to the assay must be above 33%. The radioactivity measured without enzyme must be below 8000 dpm. IC_{50} were calculated using the program NLIN SAS by non linear fitting to the equation $dpm = a/[1 + (x/IC_{50})^{nH}] + c$ defining a classical sigmoid curve where a and c represent respectively the maximum and the minimum dpm plateau values and x and IC_{50} represent respectively the concentrations (mol/L) and the IC_{50} of the studied molecules; nH is the Hill number. Starting solutions of drugs to be tested were made in dimethyl sulfoxide. These solutions were diluted with buffer containing Tris-HCl pH 8, 50 mM, $MgCl_2$, 1 mM and dithiothreitol, 5 mM, so that dimethyl sulfoxide final concentration was in any case equal to 0.1% v/v.

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