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Synthesis and biological evaluation of neutrophilic inflammation inhibitors

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Abstract

In several non-infectious human diseases, such as ulcerous colitis, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), the extravasal recruitment of neutrophils plays a crucial role in the development of tissue damage, which, when persistent, can lead to the irreversible organ dysfunction. The neutrophil activation is controlled by a number of intracellular pathways, particularly by a cAMP-dependent protein kinase A (PKA) which also acts on phosphodiesterase IV (PDE4) gene stimulating the synthesis of this enzyme, able to transform cAMP to inactive AMP. PDE4 inhibitors enhance intracellular cAMP and decrease inflammatory cell activation. Several 3-cyclopentyloxy-4-methoxybenzaldehyde and 3-cyclopentyloxy-4-methoxybenzoic acid derivatives were synthesized and studied by us to evaluate their ability to inhibit the superoxide anion production in human neutrophils. These compounds were found able to inhibit the neutrophil activation and some of them increased the cAMP level on tumor necrosis factor-α-stimulated neutrophils. Moreover, they also inhibited selectively the human PDE4 enzyme, although they are less potent than the reference compound Rolipram. We report here synthesis, biological studies and some SAR considerations concerning the above mentioned compounds.

Keywords: 3-Cyclopentyloxy-4-methoxybenzaldehyde derivatives; 3-Cyclopentyloxy-4-methoxybenzoic acid derivatives; Neutrophilic inflammation; Neutrophil inhibitors; PDE4 inhibitors

1. Introduction

The hallmark of inflammatory responses is represented by the extravascular recruitment of inflammatory cells [1,2]. Among these cells, neutrophils predominate in common forms of inflammation, such as those occurring as a consequence of tissue invasion by microorganisms and those related to immune disorders or non-specific tissue injury [3,4]. These conditions include many human diseases, such as ulcerous colitis, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), gouty arthritis and also some dermatites as psoriasis, or some vasculitis. In these non-infectious diseases, extravasated neutrophils play a crucial role in the development of tissue damage, which, when persistent, can lead to the irreversible demolition of normal tissue architecture with consequent organ dysfunction [5,6].

In inflamed tissues, neutrophils live in a microenvironment rich of pro-inflammatory substances, such as cytokines, chemotactic factors and immuno-complexes, which can effectively induce a full cell activation [7]. This activation leads to a rapid and impressive consumption of oxygen by a membrane oxidase, NADPH oxidase, which reduces the oxygen to superoxide anion (O^{2-}) giving the well-known "respiratory-burst" [8]. A number of oxidants, such as superoxide anion or hypochlorous acid (HOCl), spring up and are released extracellularly. Neutrophils activation also causes the release of potential histo-damaging enzymes from cytoplasmatic granules [6]. Oxidants, particularly HOCl, and enzymes provoke a damage of inflamed tissue and enhance the inflammatory response by their synergic pro-inflammatory activities.

Intensity and length of inflammatory response [9,10] are controlled by a cAMP dependent protein kinase A (PKA). High levels of cAMP activate PKA which phosphorylates some cytoplasmatic proteins with consequent inhibition of

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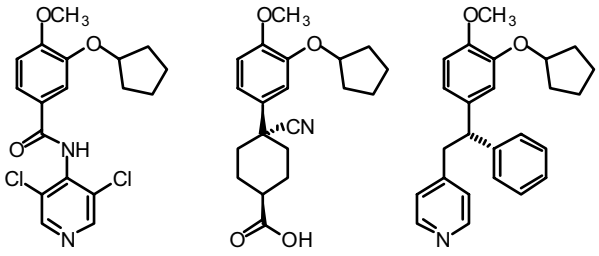
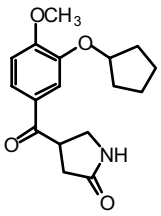
chemotaxis, degranulation and oxidative neutrophil metabolism [11]. Moreover, PKA also acts on phosphodiesterase IV (PDE4) gene [9,11] stimulating the synthesis of this enzyme, able to transform cAMP to inactive AMP. PDE4 inhibitors enhance intracellular cAMP and decrease inflammatory cells activation [12–14]. Consequently, in the last years the interest for substances able to inhibit PDE4 activation is substantially increased [15–17].

PDE4, present in the immune and inflammatory cells, is the most diffuse and, likely, the most important and selective cAMP hydrolyzing enzyme [18]. Several different structures have been reported and patented as PDE4 inhibitors [19–22], the first of which being Rolipram [23], unsuitable for therapeutic use owing to its peripheral (nausea, gastric irritation) and central (strong sedative action) side effects. It was clearly demonstrated that emesis [24], gastric acid secretion [25], and psychotropic activity [26], are linked to inhibition of the high affinity Rolipram binding site (HARBS), and that an improvement of therapeutic index can be obtained with a low PDE4 inhibition/Rolipram binding site affinity (RBSA) ratio.





Rolipram

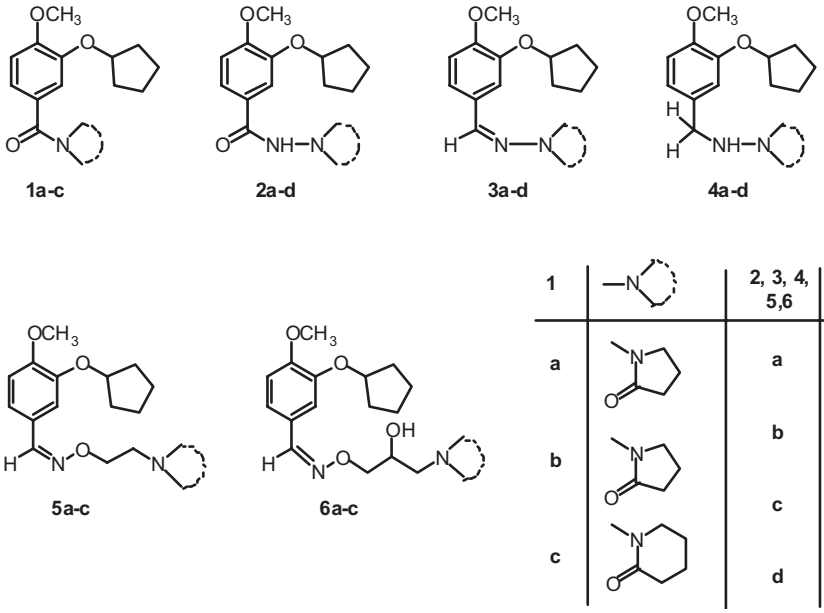
The design of new PDE4 inhibitors remained empirical for a long time, assisted in part by interesting studies of molecular modeling aimed at defining the pharmacophoric model of PDE4 catalytic site [27–30]. Recently, Xu et al. [31] determined the three-dimensional structure of the catalytic domain of PDE4B2B providing useful framework for a rational design of more selective PDE4 inhibitors and for the understanding of their mechanism of action.

In the contest of our studies on anti-inflammatory drugs [32–34], with the aim of selecting new leads for the development of compounds active in the neutrophilic inflammation, we designed and synthesized several series of Rolipram related structures in which we maintained the catechol ring with cyclopentyl and methyl substituents, regarded as crucial part for the interaction with a lipophilic pocket of the catalytic site [21] and present in several "second-generation" PDE4 inhibitors such as RP-73401 [35], Ariflo (Cilomilast) [36], CDP-840 [37].

In our compounds some simple non-aromatic nitrogen heterocycles have been linked to the catechol moiety by different functions. In particular, in the first two series **1a–c** and **2a–d**, the 2-pyrrolidinone, 2-piperidinone, 2-imidazolidinone cycles and some cycloamines, have been bonded to the aromatic ring by an amide function; in a third group of compounds, **3a–d** and **4a–d**, this function has been replaced by an imine (planar) or amine (non-planar) bond, respectively, in order to verify the role of the carbonyl group, as well as the disposition of the heterocycle moiety with respect to the phenyl ring. Finally, we have synthesized compounds **5a–c** and **6a–c**, in which heterocycle rings were spaced out from the catechol ring by more long and flexible iminoether alkyl chains.

2. Chemistry

Compounds **1a–c** and **2a–d** were prepared starting from 3-cyclopentyloxy-4-methoxy benzoic acid (**7**) (obtained from isovanilline by alkylation and subsequent oxidation [38]) which was transformed into the corresponding acid chloride **8** with thionyl chloride and then treated with 2-pyrrolidinone, 2-imidazolidinone, δ-valerolactame or the appropriate *N*-aminocycloamine in CH_2Cl_2, in the presence of anhydrous triethylamine, as shown in Scheme 1. In two cases we obtained a little amount of byproducts (the anhydride **9** and the isomer **10**, respectively) which were separated by recrystallization. In the case of compound **1b** we used 2-imidazolidinone and acid chloride **8** in the 2:1 ratio, without triethylamine in order to avoid the formation of the double acylation product. For compounds **3a–d** the 3-cyclopentyloxy-4-methoxybenzaldehyde (**11**) [38] was condensed with the appropriate *N*-aminocycloamines in anhydrous toluene using a Dean-Stark apparatus to remove water as azeotropic mixture (see Scheme 2). We used an alternative synthetic procedure to prepare the **3a–d** reduction products **4a–d**. We carried out the reductive amination of 3-cyclopentyloxy-4-methoxybenzaldehyde (**11**) with sodium cyanoborohydride in THF/ethanol mixture, in the presence of zinc chloride [39]. Then, a further addition of sodium cyanoborohydride and acidification of the reaction mixture to pH 3.8 [40] completely reduce the intermediate imino derivatives **3a–d** (quantitatively and easier formed with respect to the previous method, but not isolated in this case). Compounds **5a–c** and **6a–c** were prepared starting from 3-cyclopentyloxy-4-methoxybenzaldehyde oxime (**12**), obtained from 3-cyclopentyloxy-4-methoxybenzaldehyde (**11**) and hydroxylamine hydrochloride. This compound, reported in literature as yellow oil [41] was obtained by us, through some modifications of the reaction workup, as a white, flaky, low-melting solid (the structure was confirmed by IR and ^1H NMR spectral data). Oxime **12**, as sodium salt, was then



condensed with the appropriate ω-chloroethylamines, to obtain compounds **5a–c**. The condensation of the same sodium salt with epichlorohydrin produced the intermediate epoxide **13**, which was reacted, without purification, with the suitable cycloamines to give compounds **6a–c** (see Scheme 3).

3. Biological studies

All synthesized compounds were studied to evaluate their ability to inhibit the superoxide anion production tumor necrosis factor-α (TNFα) induced, in human neutrophils, in comparison with Rolipram as reference compound. When placed in fibronectin-coated wells, in absence of stimuli, neutrophils released detectable amount of superoxide anion (O^{2-}) up to 0.5 nmol/5 × 10^4 neutrophils during 3 h of incubation. If added at the beginning of the incubation, the addition of TNFα induced a prolonged (1.5–2 h) production of O^{2-} (3.1 ± 0.2 nmol O^{2-}/5 × 10^4 neutrophils). When Rolipram was added to the system, dose-dependent inhibition of the production of O^{2-} was observed (IC_{50} 1.43 ± 1.32 μM).

The synthesized compounds were investigated for their ability to interfere with the production of superoxide anion in the same test. All compounds, with the exception of **3c** and **4c**, inhibited in a dose-dependent way the production of O^{2-}. We reported in Table 1 the results, expressed as IC_{50} (mean of two or more experiments), for tested compounds and Rolipram.

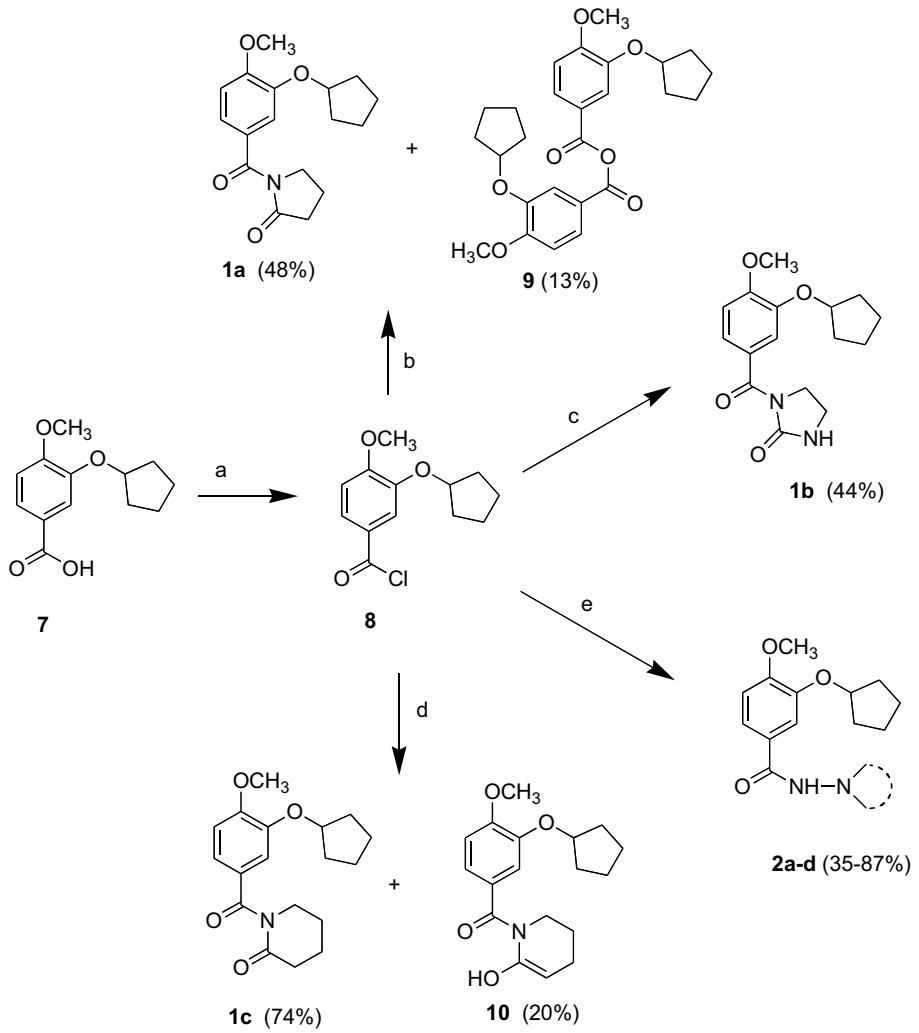
In addition, with the aim of verifying whether the lesser superoxide anion production was actually related to an inhibition of PDE4, we selected three compounds (**1a, 2a** and **3a**) and we determined the intracellular level of cAMP, in neutrophils adherent to fibronectin and TNFα stimulated, in the presence and in the absence of the substances and Rolipram, as reference compound. Rolipram, as well as tested compounds, provoked a dose-dependent increase of intracellular cAMP level as reported in Table 2 and in Fig. 1.

A further study was performed with the aim of investigating effects and selectivity of the same compounds on the human phosphodiesterases 3, 4 and 5. The effects of the tested compounds on the activities of the studied phosphodiesterases are reported in Table 3.

Moreover, in order to obtain useful indications regarding the propensity to induce side effects, we evaluated, for compound **6c**, the affinity for the HARBS. We report the competition curve obtained with **6c** at the Rolipram receptor in Fig. 2, the IC_{50} for RBA and PDE4/RBA ratio in Table 3.

4. Results and discussion

No compounds, at the concentrations used in the present work, show cytotoxic action towards the neutrophils. The percentage of vital cells never results lower than 90%. The results of the superoxide anion assay (see Table 1) suggest the following remarks. In all series screened, with the exception of **6a–c**, the products having a five-membered heterocycle linked to the catechol moiety are the most active (**1a, 2a, 3a, 4a** and **5a**), and the activity do not substantially change if a second heteroatom is present in the ring (see **1a** and **1b**). Compounds with six-membered heterocycles appear less active and show a further decrease of activity if one more heteroatom is introduced. This is particularly evident in compounds **3b, 4b** and **5b** in comparison with **3c–d, 4c–d** and **5c**, respectively. Moreover, comparing the series **2, 3, 4**, we can assert that the activity level is independent from the type of functionality linking the heterocycle to the phenyl



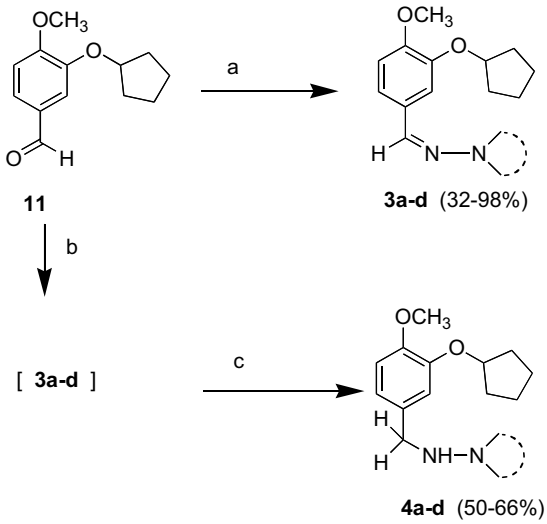
[a]Reagents: (a) $SOCl_2$; (b) 2-Pyrrolidinone, CH_2Cl_2, Et_3N; (c) 2-Imidazolidinone 2 equiv., CH_2Cl_2; (d) δ -valerolactame, CH_2Cl_2, Et_3N; (e) N-aminocycloamines, CH_2Cl_2, Et_3N.

Scheme 1. Synthetic route for the preparation of compounds **1a–c** and **2a–d**.

ring. Thus, neither the carbonyl group of the amide nor the double bond of imine function play an important role in the action. Compounds **5** result generally less active than compounds **1, 2, 3** and **4**, suggesting that spacing out the heterocyclic ring from the catecholic moiety by an iminoether chain is detrimental for the activity. Compounds of the series **6a–c** present a different behavior from all the previous ones. In these compounds the inhibiting activity reverts to the level of compounds of the series **1, 2, 3** and **4**, in spite of the spacer lengthening; furthermore, in this case, the morpholinyl derivative **6c** is the most active, while in the preceding series the morpholine ring causes a drastic decrease of activity (see particularly **3c** and **4c**). Therefore, we can suppose that, in compound **6c**, could develop a conclusive role both the oxygen atom of the morpholine ring and the hydroxy group of the iminoether chain.

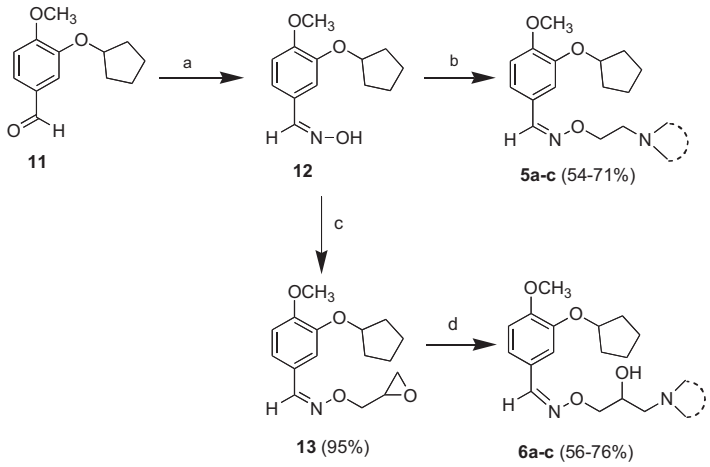
Moreover, compounds **1a, 2a** and **3a** induce a dose-dependent increase of intracellular cAMP level in neutrophils adherent to fibronectin and TNFα stimulated (see Table 2). The close correlation between this activity and the inhibition of the superoxide anion production (IC_{50}), in compounds **1a, 2a** and **3a**, suggests that they act on the neutrophils either by stimulating the cAMP production or inhibiting its hydrolysis (see Fig. 1).

The latter hypothesis seems to be confirmed by the enzymatic tests on the human PDE4 for compounds **1a, 2a, 3a** and **6c**. All the four tested compounds present a fair good inhibitory activity on the PDE4 (see Table 3), the most active being compound **6c** with an IC_{50} (μM) of 1.45 ± 0.35. Anyway, all tested compounds are less active than the reference compound Rolipram, but, being almost inactive towards



[a]Reagents: (a) N-aminocycloamines, an. toluene, Dean-Stark's apparatus; (b) N-aminocycloamines, an. THF, $NaBH_3CN$, $ZnCl_2$; (c) $NaBH_3CN$, EtOH/HCl.

Scheme 2. Synthetic route for the preparation of compounds **3a–d** and **4a–d**.



[a]Reagents: (a) NH_2OH*HCl, EtOH, $NaHCO_3$; (b) 1: EtONa/EtOH, 2: 2-Chloroethylcycloamines, an.DMF; (c) 1: EtONa/EtOH, 2: Epichlorohydrin, an. DMF; (d) Cycloamines, an. toluene.

Scheme 3. Synthetic route for the preparation of compounds **5a–c** and **6a–c**.

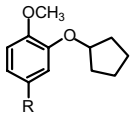
PDE3 and PDE5, they could be considered selective PDE4 inhibitors.

Finally, the selected compound **6c** specifically binds to the Rolipram receptor (see Fig. 2), but it posses about 280 times weaker affinity than Rolipram for [^{3}H]Rolipram binding site (IC_{50} = 0.59 μM vs. 0.0021 μM and Ki = 0.3 μM vs. 0.001 μM), with a low PDE4/RBSA ratio (60-fold lower than Rolipram).

5. Conclusions

In conclusion, several new Rolipram-related compounds were synthesized and tested for their ability to inhibit the superoxide anion production, TNFα induced, in human neutrophils. All compounds, except **3c** and **4c**, were able to inhibit neutrophils activation, chiefly those with a five-membered heterocycle linked to the catechol moiety. However, no correlation seems to be between the activity and the type of function that links the heterocycle to the phenyl ring. The most active compounds **1a, 2a** and **3a** showed also a good capability of increasing the cAMP level on neutrophils TNFα stimulated. The same compounds and **6c** also inhibited the human PDE4 activity. These evidences allow to affirm that the activity on the neutrophils of the synthesized compounds is due to an intracellular cAMP increase, caused in turn by the PDE4 inhibition. Finally, the most active compound **6c** showed a low PDE4/RBSA ratio, suggesting a

Table 1
Compounds **1a–c, 2a–d, 3a–d, 4a–d, 5a–c, 6a–c** and their effect on superoxide anion production, in human neutrophils (5×10^4/plate). Results are expressed as IC_{50} (mean values of two or more experiments)



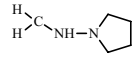
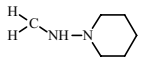
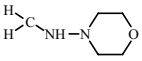
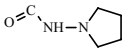
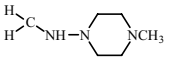
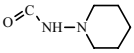
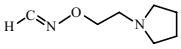
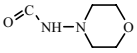
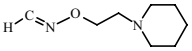
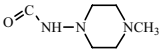
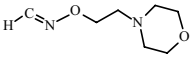
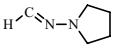
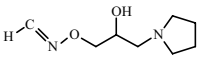
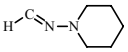
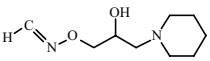
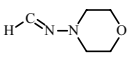
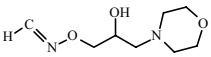
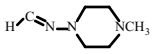
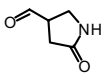
Comp	R	IC_{50} (∝M)	Comp.	R	IC_{50} (∝M)
1a		12.64 ±3.20	**4a**		11.62±3.73
1b		14.34±6.12	**4b**		22.23±4.68
1c		20.71±3.22	**4c**		Inactive
2a		10.28±3.06	**4d**		112.71±11.30
2b		23.84=12.18	**5a**		52.22±8.29
2c		28.13±9.70	**5b**		68.68±5.99
2d		27.71±2.96	**5c**		295.79±10.17
3a		12.14±6.60	**6a**		31.35±13.58
3b		17.23±6.19	**6b**		21.20±8.44
3c		Inactive	**6c**		16.30±7.39
3d		116.80±12.63	**Rolipram**		1.43±1.32

Table 2
Effect of compounds **1a, 2a** and **3a** and Rolipram, as reference compound, on intracellular cAMP level in neutrophils (10^6/plate) adherent to fibronectin and TNFα stimulated. The results are expressed as EC_{50}

Compound	EC_{50} (μM)
1a	13.44
2a	11.46
3a	6.17
Rolipram	1.14

possible lack of side effects in vivo. In addition, we must underline that compound **6c** was synthesized and tested as racemic form. As the hydroxy group, indicated as possible responsible of the activity, is just bonded to a chiral center in the iminoether chain, we cannot exclude the existence of an eutomeric form. Thus, we consider compound **6c** an interesting starting point to develop new molecules in which the rational modification of the aromatic moiety linked to the morpholinyl–hydroxypropyl–iminoether chain, as well as a stereoselective synthesis, could give new potent PDE4 inhibitors with reduced side effects.

6. Experimental section

6.1. Chemistry: materials and methods

All chemicals were obtained from Sigma Aldrich srl (Milan, Italy). Melting points are uncorrected and were measured with a Büchi 530 instrument. IR spectra were recorded with a Perkin-Elmer 398 spectrophotometer. ^{1}H NMR were recorded on a Varian Gemini 2 (200 MHz) instrument; chemical shifts are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal standard; signals were characterized as s (singlet), d (doublet), t (triplet), m (multiplet), br s (broad signal); *J* in Hz. Reactions were followed by TLC on Kieselgel $60F_{254}$ (DC-Alufolien, E. Merck, Darmstadt, Germany). Analyses for C, H, N (±0.4% of the theoretical value), were determined with an elemental analyzer EA 1110 (Fison-Instruments, Milan, Italy).

*6.1.1. Preparation of 3-(cyclopentyloxy)-4-methoxybenzoyl chloride (**8**)*

Thionyl chloride (3.6 ml, 50 mmol) was added to 3-(cyclopentyloxy)-4-methoxy-benzoic acid **7** [38], (2.36 g,

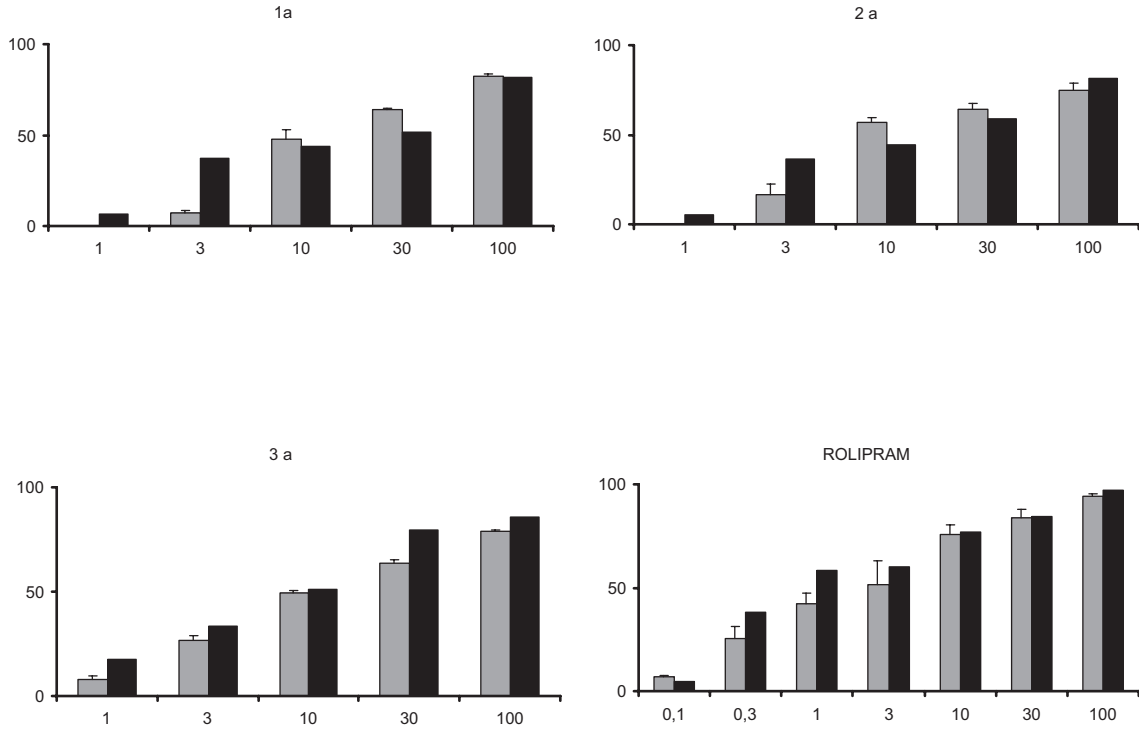


Fig. 1. Dose-dependent effect of compounds **1a, 2a, 3a** and Rolipram (μM) as % inhibition of superoxide anion production (gray bars) (mean ± 1 S.D. of two experiments) and % cAMP accumulation (black bars, results of a single experiment) in TNFα stimulated polymorphonuclear leukocytes.

Table 3
Results of PDE enzyme assay for compounds **1a, 2a, 3a, 6c**. The results are expressed as a percent inhibition of control activity (mean values; $n = 2$) or as IC_{50}

Compound	PDE3	PDE5	PDE4	RBA	PDE4/RBA
	% Inhibition (10 μM)	% Inhibition (10 μM)	IC_{50} (μM)	IC_{50} (μM)	
1a	†[a]	†	2.7 ± 0.42	nt[b]	–
2a	23	†	4.65 ± 0.07	nt	–
3a	†	†	2.85 ± 0.07	nt	–
6c	†	22	1.45 ± 0.35	0.59	2.46
Rolipram	–	–	0.32	0.0021	152

[a] The symbol † indicates an inhibition lesser than 10%.
[b] nt, not tested.

10 mmol) and the reaction mixture was stirred at 60–80 °C for 2 h. The excess thionyl chloride was evaporated under reduced pressure to give 3-(cyclopentyloxy)-4-methoxybenzoyl chloride (**8**) as a yellow oil which, after washing with ethyl ether, crystallizes by standing (yield: 1.90 g, 75%). Compound **8** has been used crude in all the following reactions.

*6.1.2. Preparation of 1-[3-(cyclopentyloxy)-4-methoxybenzoyl]pyrrolidin-2-one (**1a**)*

A solution of **8** (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 ml) was added to a solution of 2-pyrrolidinone (0.68 g, 8 mmol) and anhydrous triethylamine (1.5 ml) in CH_2Cl_2 (25 ml). The reaction mixture was stirred at 60–80 °C for 6 h and, after cooling, washed with water (20 ml) and dried ($MgSO_4$). After evaporation under reduced pressure of the solvent the obtained crude yellow oil (2.37 g) was purified by flash-chromatography on Florisil® (100–200 Mesh) with CH_2Cl_2 as eluent. From the ether solution of the purified oil a by-product crystallizes as white solid which was recrystallized from ethyl acetate. It corresponds to 3-(cyclopentyloxy)-4-methoxybenzoic anhydride (**9**), as confirmed by IR and ^1H NMR spectra and elemental analysis. The desired compound **1a** was recovered from the ether solution by evaporation of the solvent and distillation in vacuo of the oily residue (b.p.: 195 °C/0.1 mmHg). Finally, the obtained viscous oil was crystallized from ethyl ether/petroleum ether (1:1). Yield: 1.1 g, 48%, m.p. 58–60 °C. ^1H NMR ($CDCl_3$) δ 1.20–2.10 (m, 8H), 2.10–2.40 (m, 2H), 2.40–2.90 (m, 2H), 3.91 (s, 3H), 4.60–5.10 (m, 1H), 6.70–7.10 and 7.20–7.50 (2m, 3H). IR ($CHCl_3$) cm^{-1} 1745, 1665, 1270. Anal. Calcd. for

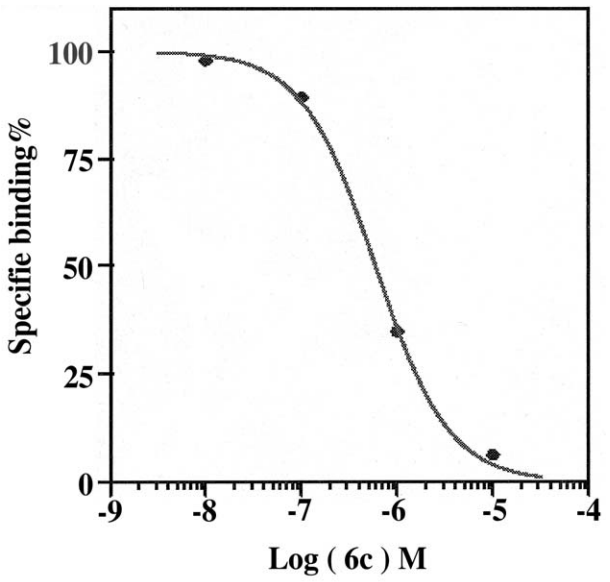


Fig. 2. Competition curve obtained with compound **6c** at the Rolipram receptor.

$C_{17}H_{21}NO_4$: C, 67.31; H, 6.98; N, 4.62. Found: C, 66.94; H, 6.86; N, 4.28.

*6.1.2.1. 3-(Cyclopentyloxy)-4-methoxybenzoic anhydride (**9**).* Yield 0.45 g, 13%, m.p. 148–149 °C, ^{1}H NMR ($CDCl_3$) δ 1.20–2.10 (m, 16H, $8CH_2$ cyclop.), 3.93 (s, 6H, $2OCH_3$), 4.85 (mc, 2H, 2CH), 6.92 (d, J = 8, 2H, Ar), 7.61 (s, 2H, Ar), 7.74 (d, J = 8, 2H, Ar). IR ($CHCl_3$) cm^{-1} 1710 (C=O), 1775 (C=O), 1265 (C–O). Anal. Calcd. for $C_{26}H_{30}O_7$: C, 68.71; H, 6.65. Found: C, 68.37; H, 6.84.

*6.1.3. Preparation of 1-[3-(cyclopentyloxy)-4-methoxybenzoyl]imidazolidin-2-one (**1b**)*

A solution of **8** (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 ml) was added dropwise to a solution of 2-imidazolidinone (1.29 g, 15 mmol) in CH_2Cl_2 (25 ml). The reaction mixture was stirred at 60–80 °C for 6 h and, after cooling, washed with water (20 ml). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to give an oil which crystallized with ethyl ether. The crude solid was recrystallized from ethyl acetate to give compound **1b** as white pure solid. Yield 1.0 g, 44%, m.p. 127–129 °C, ^{1}H NMR ($CDCl_3$) δ 1.34–2.44 (m, 8H), 3.49 (t, J = 7.2, 2H), 3.90 (s, 3H), 4.21 (t, J = 7.2, 2H), 4.60–5.00 (m, 1H), 6.32 (s, 1H, disappears with D_2O), 6.74–7.06 and 7.20–7.75 (2m, 3H). IR ($CHCl_3$) cm^{-1} 3470, 1745, 1660, 1260. Anal. Calcd. for $C_{16}H_{20}N_2O_4$: C, 63.14; H, 6.62; N, 9.20 Found: C,63.28; H, 6.83; N, 9.39.

*6.1.4. Preparation of 1-[3-(cyclopentyloxy)-4-methoxybenzoyl]piperidin-2-one (**1c**)*

A solution of **8** (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 ml) was added dropwise to a solution of δ-valerolactame (0.8 g, 8 mmol) and anhydrous triethylamine (1.5 ml) in CH_2Cl_2 (25 ml). The reaction mixture was stirred at 60–80 °C for 6 h and, after cooling, washed with water (20 ml). The organic phase was dried ($MgSO_4$) and, after evaporation under reduced pressure of the solvent, an oil (3.43 g) was obtained which crystallizes with ethyl ether. The white–pink solid obtained was then recrystallized from ethyl acetate to get desired compound **1c**. From the filtered ether solution a further white precipitate, soluble in 1 M NaOH solution and different from compound **1c** [TLC: $CHCl_3$/CH_3OH (9:1)] was obtained. It was recrystallized from ethyl acetate and resulted to be 1-[3-(cyclopentyloxy)-4-methoxybenzoyl]-1,4,5,6-tetrahydropyridin-2-ol (**10**), as confirmed by IR, ^{1}H NMR spectra and elemental analysis. Compound **1c**: yield 2.36 g, 48%, m.p. 100–101 °C. ^{1}H NMR ($CDCl_3$) δ 1.46–2.20 (m, 12H), 2.30–3.80 (m, 2H), 3.59–4.01 (m, 5H), 4.60–5.00 (m, 1H), 6.65–7.00 and 7.10–7.40 (2m, 3H). IR ($CHCl_3$) cm^{-1} 1708, 1675, 1265. Anal. Calcd. for $C_{18}H_{23}NO_4.0.5H_2O$: C, 66.24; H, 7.41; N, 4.29. Found: C, 66.42; H, 7.79; N, 4.27.

*6.1.4.1. 1-[3-(Cyclopentyloxy)-4-methoxybenzoyl]-1,4,5,6-tetrahydropyridin-2-ol (**10**).* Yield 0.73 g, 31%, m.p. 126–128 °C. ^{1}H NMR ($CDCl_3$) δ 1.50–2.10 (m, 10H, 4 CH_2-cyclop. + CH_2-piper.), 2.43 (t, J = 7.5, 2H, CH_2-piper.), 3.40–3.54 (m, 3H, CH_2-N-piper. + OH, 1H disappears with D_2O), 3.87 (s, 3H, OCH_3), 4.80–4.91 (m, 1H, CH-cyclop.), 6.24–6.37 (m, 1H, CH-piper.), 6.84 (d, J = 8.5, 1H, Ar), 7.27 (d, J = 8.5, 1H, Ar), 7.41 (s, 1H, Ar). IR ($CHCl_3$) cm^{-1} 3460, 3380 (OH), 1710 (C=O), 1645 (C=C–N), 1270 (C–O). Anal. Calcd. for: C, 64.46; H, 7.51; N, 4.18. Found: C, 64.33; H, 7.50; N, 4.17.

*6.1.5. General procedure for the preparation of 3-(cyclopentyloxy)-4-methoxy-*N*-cycloamine-1-ylbenzamides (**2a–d**)*

A solution of **8** (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 ml) was added dropwise to a solution of the appropriate *N*-aminocycloamines (8 mmol) and anhydrous triethylamine (1.5 ml) in CH_2Cl_2 (25 ml). Only in the case of compound **2a** anhydrous triethylamine (1 ml) was added to a suspension of 1-amino-pyrrolidine hydrochloride (1.48 g, 12 mmol) in CH_2Cl_2 (10 ml), the mixture was stirred at room temperature for 15 min, next a further amount of anhydrous triethylamine (1 ml) and a solution of **8** (2.31 g, 9 mmol) in CH_2Cl_2 (25 ml) were added dropwise.

In all cases the reaction mixture was then stirred at 60–80 °C for 6 h and, after cooling, washed with water (20 ml). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to yield white solids which were recrystallized from ethyl acetate or ethyl ether/petroleum ether (1:0.5) in the case of **2a**.

*6.1.5.1. 3-(Cyclopentyloxy)-4-methoxy-*N*-pyrrolidin-1-ylbenzamide (**2a**).* Yield 58%; m.p. 131–132 °C. ^{1}H NMR ($CDCl_3$) δ 1.40–2.30 (m, 12H), 2.80–3.25 (m, 4H), 3.88 (s, 3H), 4.60–5.00 (m, 1H), 6.65–7.60 (m, 4H, 1H disappears with D_2O). IR ($CHCl_3$) cm^{-1} 3000–2760, 1660, 1260. Anal. Calcd. for $C_{17}H_{24}N_2O_3$: C, 67.08; H, 7.95; N, 9.20. Found: C, 66.82; H, 8.04; N, 9.12.

6.1.5.2. 3-(Cyclopentyloxy)-4-methoxy-N-*piperidin-1-ylbenzamide (**2b**).* Yield 35%; m.p. 154–156 °C. ^{1}H NMR ($CDCl_3$) δ 1.10–2.20 (m, 14H), 2.75–3.15 (m, 4H), 3.88 (s, 3H), 4.70–5.00 (m, 1H), 6.72–7.05 and 7.25–7.65 (2m, 4H, 1H disappears with D_2O). IR ($CHCl_3$) cm^{-1} 3460–3150, 1660, 1260. Anal. Calcd. for $C_{18}H_{26}N_2O_3$: C, 67.90; H, 8.23; N, 8.80. Found: C, 67.71; H, 8.30; N, 8.80.

6.1.5.3. 3-(Cyclopentyloxy)-4-methoxy-N-*morpholin-4-ylbenzamide (**2c**).* Yield 87%; m.p. 169–170 °C. ^{1}H NMR ($CDCl_3$) δ 1.60–2.10 (m, 8H), 2.80–3.11 (m, 4H), 3.80–4.05 (m, 4H), 3.89 (s, 3H), 4.60–5.00 (m, 1H), 6.70–7.10 and 7.20–7.50 (2m, 4H, 1H disappears with D_2O). IR ($CHCl_3$) cm^{-1} 2900–2800, 1670, 1265. Anal. Calcd. for $C_{17}H_{24}N_2O_4$: C, 67.73; H, 7.55; N, 8.74. Found: C, 63.40; H, 7.72; N, 8.91.

6.1.5.4. 3-(Cyclopentyloxy)-4-methoxy-N-*(4-methylpiperazin-1-yl)benzamide (**2d**).* Yield 55%; m.p. 176–178 °C. ^{1}H NMR ($CDCl_3$) δ 1.10–2.15 (m, 8H), 2.32 (s, 3H), 2.45–2.80 and 2.80–3.25 (2m, 8H), 3.90 (s, 3H), 4.60–5.10 (m, 1H), 6.70–7.10 and 7.20–7.60 (2m, 4H, 1H disappears with D_2O). IR ($CHCl_3$) cm^{-1} 3140–3360, 1665, 1260. Anal. Calcd. for $C_{18}H_{27}N_3O_3$: C, 64.84; H, 8.16; N, 12.60. Found: C, 64.93; H, 8.12; N, 12.79.

6.1.6. General procedure for the preparation of N-*{[3-(cyclopentyloxy)-4-methoxyphenyl]methylene}cycloamin-1-amines (**3a–d**)*

A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde (**11**), (2.2 g, 10 mmol) in anhydrous toluene (20 ml) was added to a solution of the appropriate *N*-aminocycloamines (10 mmol) in anhydrous toluene (10 ml) and the reaction mixture was refluxed in a Dean-Stark apparatus for 10 h. After cooling, the solvent was evaporated under reduced pressure to give crude solids which were purified by flash-chromatography (Florisil®, 100–200 Mesh or Silicagel, CH_2Cl_2 as eluent) and then recrystallized from ethyl ether.

6.1.6.1. N-*{[3-(cyclopentyloxy)-4-methoxyphenyl]methylene}pyrrolidin-1-amine (**3a**).* Yield 98%, m.p. 84 °C. ^{1}H NMR ($CDCl_3$) δ 1.40–2.20 (m, 12H), 3.10–3.50 (m, 4H), 3.84 (s, 3H), 4.63–5.10 (m, 1H), 6.70–7.40 (m, 4H). IR ($CHCl_3$) cm^{-1} 2870, 1260. Anal. Calcd. for $C_{17}H_{24}N_2O_2$: C, 70.80; H, 8.39; N, 9.71. Found: C, 71.13; H, 8.45; N, 9.83.

6.1.6.2. N-*{[3-(cyclopentyloxy)-4-methoxyphenyl]methylene}piperidin-1-amine (**3b**).* Yield 50%, m.p. 65–67 °C. ^{1}H NMR ($CDCl_3$) δ 1.10–2.20 (m, 14H), 2.90–3.30 (m, 4H), 3.84 (s, 3H), 4.65–5.05 (m, 1H), 6.60–7.10 and 7.10–7.62 (2m, 4H). IR ($CHCl_3$) cm^{-1} 2860, 1685, 1260. Anal. Calcd. for $C_{18}H_{26}N_2O_2$: C, 71.49; H, 8.67; N, 9.26. Found: C, 71.34; H, 8.81; N, 9.40.

6.1.6.3. N-*{[3-(cyclopentyloxy)-4-methoxyphenyl]methylene}morpholin-4-amine (**3c**).* Yield 71%, m.p. 107–108 °C. ^{1}H NMR ($CDCl_3$) δ 1.45–2.20 (m, 8H), 3.00–3.32 (m, 4H), 3.70–4.10 (m, 7H), 4.65–5.10 (m, 1H), 6.70–7.10 and 7.20–7.80 (2m, 4H). IR ($CHCl_3$) cm^{-1} 2860, 1665, 1260. Anal. Calcd. for $C_{17}H_{24}N_2O_3$: C, 67.08; H, 7.95; N, 9.20. Found: C, 66.82; H, 7.78; N, 9.25.

6.1.6.4. N-*{[3-(cyclopentyloxy)-4-methoxyphenyl]methylene}-4-methylpiperazin-1-amine (**3d**).* Yield 32%, m.p. 78–80 °C. ^{1}H NMR ($CDCl_3$) δ 1.33–2.15 (m, 8H), 2.36 (s, 3H), 2.46–2.80 (m, 4H), 3.00–3.40 (m, 4H), 3.85 (s, 3H), 4.60–5.05 (m, 1H), 6.70–7.10 and 7.10–7.65 (2m, 4H). IR ($CHCl_3$) cm^{-1} 2840, 1660, 1260. Anal. Calcd. for $C_{18}H_{27}N_3O_2$: C, 68.11; H, 8.57; N, 13.00. Found: C, 68.40; H, 8.74; N, 13.32.

6.1.7. General procedure for the preparation of N-*[3-(cyclopentyloxy)-4-methoxybenzyl]cycloamin-1-amines (**4a–d**)*

The appropriate amine (10 mmol), $NaBH_3CN$ (0.63 g, 10 mmol), $ZnCl_2$ (0.5 g, 5 mmol) were added in succession to a solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde (**11**) (2.2 g, 10 mmol) in anhydrous tetrahydrofuran (THF) (8 ml) and anhydrous ethanol (2 ml). Only in the case of compound **4a** also anhydrous triethylamine (1.5 ml) was added to release 1-amino-pyrrolidine from its hydrochloride. The reaction mixture was stirred at room temperature and after 1 h the presence of only compounds **3a–d** was detected [TLC: $CHCl_3$]. So, a further amount of $NaBH_3CN$ (0.3 g, 5 mmol) was added and the solution was made acidic (pH 3.8) by adding dropwise a saturated HCl/ethanol solution (color change of Bromocresol Green from blue to yellow). After stirring at room temperature for 12 h the solvents were evaporated under reduced pressure, the residue was dissolved in water (10 ml), the water solution made alkaline with NaOH 1 M solution and, finally, extracted three times with $CHCl_3$ (5 ml). The organic phases were dried ($MgSO_4$) and evaporated under reduced pressure to give crude oils which were purified by flash-chromatography (Florisil® 100–200 Mesh, $CHCl_3$ as eluent) and distillation under high vacuo.

6.1.7.1. N-*[3-(cyclopentyloxy)-4-methoxybenzyl]pyrrolidin-1-amine (**4a**).* Yield 63%, b.p. 180/0.8 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.50–2.20 (m, 12 H), 2.65 (bs, 1H, NH, disappears with D_2O), 3.17–3.50 (m, 4H), 3.85 (s, 3H), 3.95 (s, 2H), 4.65–5.10 (m, 1H), 6.80–7.04 and 7.10–7.34 (2m, 3H). Anal. Calcd. for $C_{17}H_{26}N_2O_2$: C, 70.31; H, 9.02; N, 9.65. Found: C, 70.16; H, 9.14; N, 9.84.

6.1.7.2. N-*[3-(cyclopentyloxy)-4-methoxybenzyl]piperidin-1-amine (**4b**).* Yield 50%, b.p. 180/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.30–2.20 (m, 15H, 1H disappears with D_2O), 3.10–3.50 (m, 4H), 3.82 (s, 3H), 4.28 (s, 2H), 4.70–5.00 (m, 1H) and 6.77–7.25 (m, 3H). Anal. Calcd. for $C_{17}H_{28}N_2O_2$.HCl: C, 63.42; H, 8.57; N, 8.22. Found: C, 63.40; H, 8.43; N, 7.98.

6.1.7.3. N-*[3-(cyclopentyloxy)-4-methoxybenzyl]morpholin-4-amine (**4c**).* Yield 66%, b.p. 190–200/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.46–2.10 (m, 8H), 2.54–2.90 (m, 4H),

3.58–4.04 (m, 10H, 1H disappears with D_2O), 4.60–4.96 (m, 1H), 6.70–7.10 and 7.15–7.65 (2m, 3H). Anal. Calcd. for $C_{17}H_{26}N_2O_3$: C, 66.64; H, 8.55; N, 9.14. Found: C, 66.30; H, 8.58; N, 9.32.

6.1.7.4. N-*[3-(cyclopentyloxy)-4-methoxybenzyl]-4-methylpiperazin-1-amine (**4d**).* Yield 57%, b.p. 190/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.52–2.10 (m, 8H), 2.35 (s, 3H), 2.47–2.77 (m, 7H, 1H disappears with D_2O), 3.06–3.36 (m, 6H), 3.84 (s, 3H), 4.70–4.96 (m, 1H), 6.65–7.05 and 7.22–7.68 (2m, 3H). Anal. Calcd. for $C_{18}H_{29}N_3O_2$: C, 67.68; H, 9.15; N, 13.15. Found: C, 67.35; H, 8.96; N, 13.00.

*6.1.8. Preparation of 3-(cyclopentyloxy)-4-methoxybenzaldehyde oxime (**12**)*

A solution of hydroxylamine hydrochloride (7.5 g, 108 mmol) in water (50 ml) was added to a solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde (**11**) (12 g, 54 mmol) in ethanol 95% (150 ml). Then, $NaHCO_3$ (9.1 g, 108 mmol), in small portions, and water (50 ml) in succession were added, and the mixture was stirred for 4 h at room temperature. Additional water (50 ml) was added and the mixture allowed to stand at –4 °C for 12 h. Finally, from the frozen suspension a white solid crystallized, which was filtered and dried at room temperature.

Yield 7.87 g, 62%, m.p. 54 °C. ^{1}H NMR ($CDCl_3$) δ 1.50–1.70 and 1.70–2.10 (2m, 8H), 3.87 (s, 3H), 4.75–4.85 (m, 1H), 6.84 (d, J = 8, 1H), 7.02 (dd, J = 8, J = 1, 1H), 7.20 (d, J = 1, 1H), 8.07 (s, 1H) and 8.30 (bs, 1H, disappears with D_2O). IR ($CHCl_3$) cm^{-1} 3580, 3480–3120, 1600, 1260. Anal. Calcd. for $C_{13}H_{17}NO_3.H_2O$: C, 61.64; H, 7.56; N, 5.53. Found: C, 62.01; H, 7.95; N, 5.64.

6.1.9. General procedure for the preparation of 3-(cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-cycloamin-1-ylethyl)oximes (**5a–c**)*

3-(Cyclopentyloxy)-4-methoxybenzaldehyde oxime (**12**) (1.18 g, 5 mmol) was added to a solution of sodium (0.12 g, 5 mmol) in anhydrous ethanol (5 ml) and the mixture was stirred for 15 min. Then, ethanol was evaporated under reduced pressure, the sodium salt of **12** was dissolved in anhydrous DMF (5 ml) and a solution of the appropriate ω-chloroethylamine, previously released from its hydrochloride, in anhydrous DMF (5 ml) was added dropwise. The reaction mixture was stirred at 40–50 °C for 12 h. After cooling to room temperature the mixture was poured into water (30 ml), extracted with $CHCl_3$ and dried ($MgSO_4$). After solvent evaporation under reduced pressure yellow oils were obtained which were purified by distillation in high vacuo.

6.1.9.1. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-pyrrolidin-1-ylethyl)oxime (**5a**).* Yield 71%, b.p. 195–200/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.40–2.16 (m, 12H), 2.60 (t, J = 8.4, 4H), 2.80 (t, J = 6, 2H), 3.84 (s, 3H), 4.30 (t, J = 6, 2H), 4.65–5.00 (m, 1H), 6.68–7.07 and 7.12–7.36 (2m, 3H), 8.08 (s, 1H). IR ($CHCl_3$) cm^{-1} 1600, 1260. Anal. Calcd. for $C_{19}H_{28}N_2O_3$: C, 68.65; H, 8.49; N, 8.43. Found: C, 68.26; H, 8.66; N, 8.70.

6.1.9.2. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-piperidin-1-ylethyl)oxime (**5b**).* Yield 54%, b.p. 190–195/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.10–2.15 (m, 14 H), 2.50 (t, J = 6, 4H), 2.70 (t, J = 6, 2H), 3.86 (s, 3H), 4.30 (t, J = 6, 2H), 4.65–5.00 (m, 1H), 6.70–7.06 and 7.11–7.30 (2m, 3H), 8.05 (s, 1H). IR ($CHCl_3$) cm^{-1} 1600, 1260. Anal. Calcd. for $C_{20}H_{30}N_2O_3$: C, 69.33; H, 8.73; N, 8.09. Found: C, 69.26; H, 9.07; N, 8.06.

6.1.9.3. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-morpholin-4-ylethyl)oxime (**5c**).* Yield 57%, b.p. 190–200/0.4 °C/mmHg. ^{1}H NMR (CDCl3) δ 1.59–2.11 (m, 8H), 2.51 (t, J = 4.8, 4H), 2.70 (t, J = 6, 2H), 2.72 (t, J = 4.8, 4H), 3.84 (s, 3H), 4.30 (t, J = 6, 2H), 4.68–4.98 (m, 1H), 6.83–7.05 and 7.18–7.32 (2m, 3H), 8.05 (s, 1H). IR ($CHCl_3$) cm^{-1} 1600, 1260. Anal. Calcd. for $C_{19}H_{28}N_2O_4$: C, 65.49; H, 8.10; N, 8.04. Found: C, 65.25; H, 8.10; N, 8.20.

6.1.10. Preparation of 3-(cyclopentyloxy)-4-methoxybenzaldehyde O-*(oxiran-2-ylmethyl)oxime (**13**).* 3-(Cyclopentyloxy)-4-methoxybenzaldehyde oxime (**12**) (2.36 g, 10 mmol) was added to a solution of sodium (0.24 g, 10 mmol) in anhydrous ethanol (10 ml) and the mixture was stirred for 15 min. Then, ethanol was evaporated under reduced pressure, the sodium salt of **12** was dissolved in anhydrous DMF (10 ml) and a solution of epichlorohydrin (1.38 g, 15 mmol) was added dropwise. The reaction mixture was stirred at 40–50 °C for 12 h. After cooling to room temperature the mixture was poured into water (20 ml), extracted with CH_2Cl_2, dried ($MgSO_4$) and the solvent evaporated under reduced pressure. The pale yellow oil obtained was purified by distillation in high vacuo and used without characterization in the following reactions. Yield 2.78 g, 95%, b.p. 200–210/0.4 °C/mmHg.

6.1.11. General procedure for the preparation of 3-(cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-hydroxy-3-cycloamin-1-ylpropyl)oximes (**6a–c**).* A suitable amine (10 ml) was added to a solution of epoxide **13** (2.91 g, 10 mmol) in anhydrous ethanol and the mixture stirred at 40–50 °C for 12 h. Then, the cooled solution was washed twice with water (10 ml) and extracted three times with 1 M HCl solution. The acid phases were made alkaline with 1 M NaOH solution and extracted with $CHCl_3$. The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to give yellow oils which were purified by distillation in high vacuo. Compound **6b** was characterized as hydrochloride.

6.1.11.1. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-hydroxy-3-pyrrolidin-1-ylpropyl)oxime (**6a**).* Yield 56%, b.p. 220–225/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.38–2.18 (m, 12 H), 2.28–2.88 (m, 6H), 3.76 (s, 1H, disappears with D_2O),

3.87 (s, 3H), 4.10–4.35 (m, 3H), 4.63–5.00 (m, 1H), 6.70–7.05 and 7.10–7.35 (2m, 3H), 8.10 (s, 1H). IR ($CHCl_3$) cm^{-1}3410, 1600, 1200–1260. Anal. Calcd. for $C_{20}H_{30}N_2O_4$: C, 66.27; H, 8.34; N, 7.73. Found: C, 66.21; H, 8.61; N, 8.01.

6.1.11.2. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-hydroxy-3-piperidin-1-ylpropyl)oxime (**6b**).* Yield 68%, b.p. 225–230/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.45–2.25 (m, 14 H), 3.09–3.39 (m, 4H), 3.53–3.70 (m, 2H), 3.73–4.00 (bs, 1H, disappears with D_2O), 3.87 (s, 3H), 4.17–4.38 (m, 3H), 4.55 (s, 1H, H_2O, disappears with D_2O), 4.56–4.98 (m, 1H), 6.75–7.07 and 7.12–7.30 (2m, 3H), 8.07 (s, 1H), 10.90–11.10 (bs, 1H, NH^+, disappears with D_2O). IR ($CHCl_3$) cm^{-1} 3500–3150, 2680–2300, 1600, 1260. Anal. Calcd. for $C_{21}H_{32}N_2O_4$.HCl.0.5H_2O: C, 59.78; H, 8.12; N, 6.64. Found: C, 59.52; H, 7.80; N, 6.86.

6.1.11.3. 3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-*(2-hydroxy-3-morpholin-4-ylpropyl)oxime (**6c**).* Yield 76%, b.p. 215–220/0.4 °C/mmHg. ^{1}H NMR ($CDCl_3$) δ 1.50–2.10 (m, 8H), 2.40–2.80 (m, 6H), 3.55 (s, 1H, disappears with D_2O), 3.76 (t, J = 4.8, 4H), 3.88 (s, 3H), 4.10–4.35 (m, 3H), 4.70–5.00 (m, 1H), 6.85–7.05 and 7.11–7.32 (2m, 3H), 8.10 (s, 1H). IR ($CHCl_3$) cm^{-1} 3600–3200, 1600, 1260. Anal. Calcd. for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 7.40. Found: C, 63.50; H, 8.03; N, 7.65.

6.2. Biology: materials and methods

Dulbecco's PBS and HBSS were purchased from Irvine Scientific. Heparin was obtained from Roche, Milano, Italy. Ficoll-Hypaque was purchased from Seromed, Berlin, Germany. Fluorescein diacetate, ethidium bromide, human albumin, ferricytochrome *c*, superoxide dismutase were obtained from Sigma-Aldrich S.r.l., Milano, Italy. Fibronectin, TNFα, Rolipram were obtained from ICN Biomedical S.r.l., Milano, Italy.

6.2.1. Neutrophil isolation

Heparinized (heparin 10 U/ml) venous blood was obtained from healthy volunteers after informed consent. Neutrophils were isolated by dextran sedimentation, subsequent centrifugation on a density gradient and removal of contaminating erythrocytes by hypotonic lysis. The resulting neutrophils were washed three times with incubation medium and final cell suspensions always containing 97% or more viable cells.

6.2.2. Neutrophil membrane integrity assay

Neutrophil viability measured as integrity of membrane was assessed according Dankberg and Perdinsky [42] as previously described. Briefly, cells (4×10^4/100 µl) harvested from culture tubes were mixed with 50 µl of staining solution (2 µg/ml fluorescein diacetate, 4 µg/ml ethidium bromide in HBSS) and incubated 10 min at room temperature. Thereafter, a drop of cell suspension was placed on a slide, sealed with a coverslip and analyzed under ultraviolet light in a dark field illumination. Neutrophils with intact membrane (i.e. viable cells) appeared as green fluorescent cells, whereas neutrophils with damaged and ethidium bromide-permeable membrane (i.e. necrotic cells) displayed a fluorescent red nucleus.

6.2.3. Superoxide anion assay (Table 1)

The production of superoxide anion by neutrophils was measured by the superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction, using a microplate reader. The assay was carried out in 96-well, flat-bottomed, polystyrene plates [43]. The wells were pretreated with 50 µl of fibronectin (1 ng/well) by incubation in 5% CO_2 at 37 °C for 2 h. After three washings with normal saline, 25 µl incubation medium containing 75 µM cytochrome *c* (final concentration) were added to each well. Then, TNFα added (10 ng/ml, final concentration) followed by 50 µl/cells (5×10^4 neutrophils). Experiments were carried out in triplicate, in the presence and absence of SOD (150 U/ml). Moreover, experiments were carried out in absence and presence of Rolipram and new synthesized compounds at concentration range of 1–300 µM. Each compound was added to wells before the addition of TNFα. The reduction of cytochrome *c* was monitored at intervals (5–15 min) by reading the plate at 550 nm. The amounts of O^{2-} produced by neutrophils were determined from the OD550 of samples without SOD minus the OD550 of matched samples with SOD, using an extinction coefficient ε 9500 l mol^{-1} cm^{-1}, calculated according to Leslie [44].

6.2.4. Intracellular cAMP assay (Table 2 and Fig. 1)

The intracellular cAMP accumulation was determined by a radioimmunoassay cAMP [^{125}I] assay system (Amersham Pharmacia Biotech). The assay was carried out in 16 mm diameter wells, flat-bottomed, polystyrene plates (Falcon 3047, Becton Dickinson) pretreated with fibronectin. Neutrophils (106 in 100 µl) were added to prewarmed incubation medium containing 10 ng/ml TNFα (final volume 500 µl). After 30 min incubation the reaction was stopped by boiling the samples for 4 min. After centrifugation at 600 *g* for 5 min, supernatants were stored at –80 °C until cAMP measurement according to the manufacturer's protocol.

6.3. Enzyme assay

All the phosphodiesterase enzyme assay were performed by CEREP (Study Director: Prof. Dupuis P., Celle L'Evescault, France), using the following standard procedures.

In all the experiments, the reference compounds were tested at nine concentrations in duplicate to obtain an inhibition curve in order to validate this experiment.

Results are expressed as a percent inhibition of control activity obtained in the presence of the test compounds. IC_{50} values and Hill coefficient (n_H) were determined for the

reference compounds by non-linear regression analysis of its inhibition curve. These parameters were obtained by Hill equation curve fitting. The IC_{50} values obtained for the reference compounds are within accepted limits of historic averages obtained ± 0.5 log unit.

6.3.1. PDE3 enzyme assay (Table 3)

PDE3 purified from human platelets [45] was incubated for 30 min at 30 °C with [^{3}H]cAMP as tracer and cAMP (0.1 μM) as substrate, in the presence of Milrinone as reference compound (IC_{50} = 0.20 μM, n_H = 1) and the tested compounds dissolved in DMSO (10^{-3} M) and diluted with water (10 μM), in duplicate. The level of reaction product [^{3}H]5′-AMP was detected with a scintillation counter (Topcount, Packard) using a liquid scintillation cocktail (Microscint 20, Packard).

6.3.2. PDE4 enzyme assay (Table 3)

PDE4 purified from human monocyte (U-937) [46] was incubated for 30 min at 30 °C with [^{3}H]cAMP as tracer and cAMP (1 μM) as substrate, in the presence of Rolipram as reference compound (IC_{50} = 0.32 μM, n_H = 0.7) and the tested compounds, dissolved in DMSO (10^{-3} M) and diluted with water (10, 1, 0.1, 0.01 and 0.001 μM), in duplicate. The level of reaction product [^{3}H]5′-AMP was detected with a scintillation counter (Topcount, Packard) using a liquid scintillation cocktail (Microscint 20, Packard).

6.3.3. PDE5 enzyme assay (Table 3)

PDE5 purified from human platelets [45] was incubated for 30 min at 30 °C with [^{3}H]cGMP as tracer and cGMP (1 μM) as substrate, in the presence of dipyridamole as reference compound (IC_{50} = 1.2 μM, n_H = 0.9) and the tested compounds dissolved in DMSO (10^{-3} M) and diluted with water (10 μM), in duplicate. The level of reaction product [^{3}H]5′-GMP was detected with a scintillation counter (Topcount, Packard) using a liquid scintillation cocktail (Microscint 20, Packard).

6.3.4. [^{3}H]Rolipram binding assay (Table 3 and Fig. 2)

A mixture consisting of (±)[^{3}H]Rolipram, test compound and mouse brain membrane preparation [24], was incubated for 60 min at 4 °C, in duplicate. Rolipram (10 mM) was used for non-specific binding. The contents of incubation tubes were filtered through a glass filter, the membranes were washed three times with ice-cold buffer, and the radioactivity on the separated disks was determined in a liquid scintillation counter. IC_{50} values were determined from semilog graphs of percent inhibition vs. concentration.

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