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Synthesis, Biological Evaluation, and Molecular Modeling of New 3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-(2-(2,6-Dimethylmorpholino)-2-oxoethyl) Oxime (GEBR-7b) Related Phosphodiesterase 4D (PDE4D) Inhibitors

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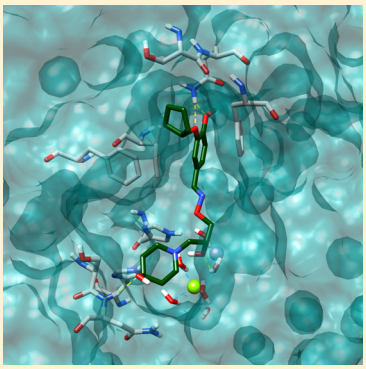
Supporting Information

ABSTRACT: A new series of 3-(cyclopentyloxy)-4-methoxyphenyl derivatives, structurally related to our hit GEBR-4a (**1**) and GEBR-7b (**2**), has been designed by changing length and functionality of the chain linking the catecholic moiety to the terminal cycloamine portion. Among the numerous molecules synthesized, compounds **8**, **10a**, and **10b** showed increased potency as PDE4D enzyme inhibitors with respect to **2** and a good selectivity against PDE4A4, PDE4B2, and PDE4C2 enzymes, without both cytotoxic and genotoxic effects. The ability to enhance cAMP level in neuronal cells was assessed for compound **8**. SAR considerations, also confirmed by in silico docking simulations, evidenced that both chain and amino terminal function characterized by higher hydrophilicity are required for a good and selective inhibitor−catalytic pocket interaction.



INTRODUCTION

The second messengers cAMP and cGMP modulate the cellular response to several hormones and neurotransmitters in the signal transduction pathway.[1]

By hydrolyzing cAMP and cGMP respectively to inactive 5′-AMP and 5′-GMP, phosphodiesterase enzymes (PDEs) regulate their intracellular levels and consequently exert a control on a number of cellular functions.[2,3] Among the large family of PDEs, the type 4 (PDE4) is specific for cAMP and ubiquitous in the body. Four different isoforms (namely PDE4A, PDE4B, PDE4C, and PDE4D) are coded by independent genes (Pde4a-Pde4d), and over 20 splice variants have been identified and classified (e.g., PDE4A2, PDE4B2, PDE4D1, PDE4D2,...).[4,5]

On the basis of their primary structures, PDE4 can be also distinguished in "long", "short", or "super-short" enzymes.[4] The "long"-isoforms are characterized by two regulatory regions (namely upstream conserved region 1 and 2, UCR1 and UCR2) inserted between the N-terminal portion and the catalytic domain. The UCR1 domain contains a site for protein kinase A (pKA) phosphorylation and is missing in the "short" forms, whereas the "supershort" forms not only lack UCR1 but also have a truncated UCR2 domain. In PDE4D UCR2 domain, a phenylalanine residue, replaced by a tyrosine in PDE4A, B, and C, has been proposed by Gurney and co-workers as a key interaction site for allosteric inhibitors.[6] More recently, an additional C terminal helix (named conserved region 3, CR3) has been identified by the same authors in a protein construct used to cocrystallize the catalytic domains of PDE4 with different small molecules active as inhibitors.[7] As different sequences have been evidenced in CR3 domain of PDE4D and PDE4B, the specific interaction with catalytic pocket and CR3 terminal domain has been suggested as a possible key point for selective inhibitors development. However, this additional information is still difficult to be used for the rational design of new compounds, due to the flexibility shown by UCR2 and CR3 domains. In fact, they are the most external parts of the protein, and in the absence of the complete 3D structure of the enzyme, which could assign them a specific region to be occupied, they are free to move in a consistent way during in silico simulation.

In the last 15 years, several strategies designed to enhance cerebral cAMP by PDEs inhibition have been proposed for the

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treatment of neurological disorders.[8−14] By preventing cAMP hydrolysis, PDE4 inhibitors (PDE4Is) enhance intracellular signal transduction and increase the phosphorylation of cAMP response element-binding protein (CREB) and the transcription of proteins related to synaptic plasticity and memory formation. However, blockade of all the different PDE4 isoforms by nonselective PDE4Is, such as Rolipram (Figure 1), combines procognitive properties with undesirable effects (particularly hypolocomotion in rodents and emesis),[15] that prevent their therapeutic use.

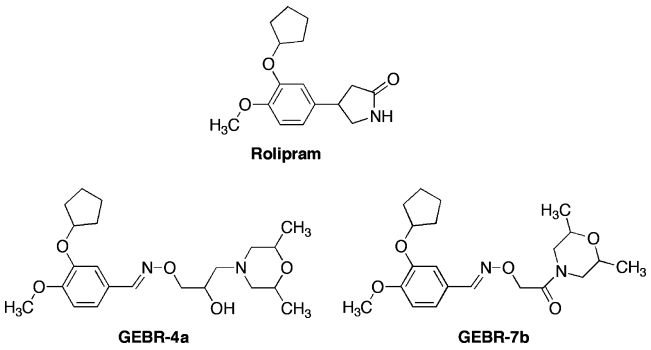

Figure 1. Structures of Rolipram and hit compounds **1** (GEBR-4a) and **2** (GEBR-7b).

Our recent research on PDE4Is led to a series of small molecules bearing a catecholic moiety (typical of Rolipram related PDE4Is) and an amino function linked to the aromatic portion by an imino-ether chain. SAR studies evidenced a pivotal role for the chain length and its spatial direction, particularly in determining the selectivity toward the different PDE4 isoforms.[16,17] Two compounds, 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(3-(2,6-dimethylmorpholino)-2-hydroxypropyl) oxime[17] (GEBR-4a, **1**, Figure 1) and 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(2-(2,6-dimethylmorpholino)-2-oxoethyl) oxime[17] (GEBR-7b, **2**, Figure 1) showed a competitive inhibition of PDE4D higher than PDE4A, PDE4B, and PDE4C.

The most active compound (**2**) was selected for additional in vitro and in vivo studies to assess its ability to modulate memory functions and to analyze possible side effects in mice and rats. Interestingly, we demonstrated that **2**, despite it was less active than Rolipram as PDE4D inhibitor in vitro, it was 10 times more potent on cognition in vivo. In addition, **2** did not induce any emetic-like effect in rats at doses up to 100 times higher than those effective on memory, as measured with the taste reactivity test. Similarly, in the xylazine/ketamine test in mice, **2** did not reduce the duration of anesthesia at doses up to 33 times higher than the procognitive dose.[18] It is noteworthy that **2** completely inhibited enzyme activity at high concentration (Figure 1, Supporting Information), differently than PDE4D allosteric inhibitor D158681.[6] Our results further supported the idea that it is possible to separate emesis from procognitive effects not only with PDE4D modulators but also with selective PDE4D inhibitors. In addition, we have also recently reported that chronic prophylactic treatment with **2** can improve spatial memory in the APPswe/PS 1dE9 mouse model of Alzheimer's disease (AD), even after pathology onset, without altering emotional or neuroendocrine regulation.[19]

Introductory molecular docking calculations evidenced that our leads **1** and **2** interact with the enzyme in similar way at the level of the catecholic system and of amino terminal group. To increase our knowledge about those pivotal enzyme interactions, we planned the synthesis of a new generation of small molecules by modifying **1** and **2** at the level of the linker from the catecholic moiety and the terminal cycloamine. In particular, we: introduced a shorter chain bearing or not a nitrogen and/or an oxygen atom (compounds **3a−c**, **4a**,**b**, **5a**,**b**, **11a−c**, and **12a−c**, Table 1), increased the length of the chain by an additional carbon or nitrogen atom (compounds **7a**,**b** and **10a**,**b**, Table 1), investigated the role of CH═N−O− moiety by replacing it with unsaturated or saturated carbon linker (compounds **11a−c**, **12a−c**, Table 1), replaced the terminal hydrogen bond acceptor (HBA) morpholine function with the 4-hydroxypiperidine moiety that can act as hydrogen bond donor (HBD) also (compounds **3c**, **6**, **8**, **11c**, **12c**, Table 1), and modified the molecule polarity by both hydroxypiperidine and hydroxypropyl moieties esterification (compound **9**, Table 1).

All the synthesized compounds were preliminarily tested in duplicate at the concentration of 10 μM on recombinant human PDE4D3 enzymes expressed in a baculoviral system (Table 1). To assess the selectivity, the most active compounds were tested at the concentration of 10 μM on PDE4A4, PDE4B2, and PDE4C2 (Table 2). Subsequently, IC_{50} values were determined only for those compounds showing PDE4D3 and PDE4B2 inhibitory activity higher than 50% (the most common cutoff value for further investigation), using Rolipram, **1**, and **2** as reference compounds (Table 3).

On the basis of their enzymatic profile (potency and selectivity), we selected compounds **5a**,**b**, **8**, and **10a**,**b** to evaluate their cyto- and genotoxicity and to assess their ability to increase cAMP levels in neuronal cells.

To rationalize the pharmacological results obtained and try to gain information on the binding mode of these derivatives, keeping into account also their selectivity, in silico docking studies were performed on all new synthesized compounds and on the hits **1** and **2**, using PDE4B and PDE4D three-dimensional models (PDB codes 1XMU and 1XOQ, respectively).

RESULTS

Chemistry. The starting 3-(cyclopentyloxy)-4-methoxybenzaldehyde **14** was obtained following a literature methods[20] from isovanilline **13** by alkylation with bromocyclopentane. By oxidation of **14** with sulphamic acid, we obtained the 3-cyclopentyloxy-4-methoxy benzoic acid **15**,[20] which was then transformed into the corresponding acid chloride with thionyl chloride and subsequently treated with the suitable cycloamine or *N*-aminocycloamine to obtain compounds **3a−c** and **4a**,**b** as reported in Scheme 1.

Compounds **5a**,**b** were obtained by treatment of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **14** with the appropriate *N*-aminocycloamine in anhydrous toluene in a Dean−Stark apparatus, as reported in Scheme 1.

For compounds **6** and **7a**,**b**, we applied a convergent synthetic strategy which first involves the transformation of **14** into the corresponding oxime **16** with hydroxylamine in ethanol.[16] **16** was obtained as a mixture of two isomers (*syn*/*anti*), the first (*syn*) being preponderant (about 75%) as previously reported.[17] The subsequent reaction of **16** with the suitable chloroacetylamine or chloropropanoylamine gave compounds **6** and **7a**,**b** as a mixture of *E*/*Z* isomers, being prevalent the *E* isomers, as reported in Scheme 1. Owing the

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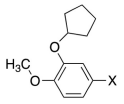
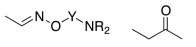
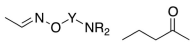
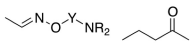
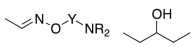
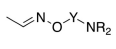
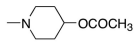
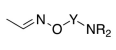
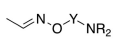
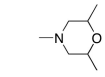
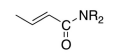
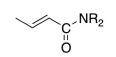
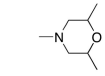
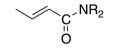
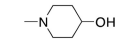
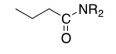
Table 1. Molecular Structure, Inhibition Activity Percent of Compounds 3a−c, 4a,b, 5a,b, 6, 7a,b, 8, 9, 10a,b, 11a−c, and 12a−c, and 1, 2, and Rolipram as Reference Compounds toward PDE4D3 Isoform[a]



Comp.	X	Y	NR_2	PDE4D3 %inib. at 10μM
3a		–		45
3b		–		31
3c		–		20
4a[b]		-		32
4b		–		0
5a[b]		–		83
5b		–		75
6				30
7a				49
7b				24
8				65

Comp.	X	Y	NR_2	PDE4D3 %inib. at 10μM
9				12
10a				56
10b				80
11a		-		25
11b		-		16
11c		-		22
12a		-		7
12b		-		10
12c		-		12
1				68
2				67
Rolipram		-	-	80

[a]The results are expressed as % inhibition in respect to the control at 10 μM concentration, in duplicate. [b]The syntheses of compounds **4a** and **5a**, but not their enzymatic profiles, have been already published.[15]

Table 2. Inhibition Activity Percent Toward Different PDE4 Isoforms (at 10 μM Concentration)[a] of Compounds 3b, 4a, 5a,b, 8, and 10a,b, and 1, 2, and Rolipram, as Reference Compounds

compd	PDE4D1 (%inhib)	PDE4D2 (%inhib)	PDE4D3 %inhib	PDE4A4 (%inhib)	PDE4B2 (%inhib)	PDE4C2 (%inhib)
3b	18	9	31	nt[b]	nt	nt
4a	49	32	32	nt	nt	nt
5a	75	62	83	41	62	31
5b	68	53	75	29	47	20
8	nt	nt	65	10	5	9
10a	nt	nt	56	33	34	20
10b	65	74	80	41	51	25
1	54	53	68	34	39	19
2	57	76	67	34	23	25
Rolipram	78	80	79	79	65	57

[a]The results are expressed as % inhibition in respect to the control at 10 μM concentration, in duplicate. [b]nt = not tested.

impossibility to resolve the mixture of *E*/*Z* isomers with classical chromatographic methods, the relative quantities of the isomers was not calculated. The same synthetic procedure was applied, with a strong improvement on the yield, to the synthesis of compound **2**, previously obtained by a less profitable step-by-step reaction.[17]

Compounds **8** and **10a,b** were obtained by reacting the suitable cycloamine or *N*-aminocycloamine with 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(oxiran-2-ylmethyl)-oxime **17**, in turn prepared from 3-(cyclopentyloxy)-4-methoxybenzaldehyde oxime **16** with epichlorohydrin.[16] The acetyl derivative **9** was obtained reacting **8** with acetic anhydride as reported in Scheme 1.

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Table 3. IC_{50} Values (μM)[a] toward PDE4D3 and PDE4B2 of Compounds 5a,b, 8, and 10a,b, and 1, 2 and Rolipram, as Reference Compounds

compd	PDE4D3 (IC_{50} μM)	PDE4B2 IC_{50} (μM)
5a	0.66	1.6
5b	1.55	nt[b]
8	1.79	nt
10a	1.26	nt
10b	0.21	18.0
1	3.46	nt
2	1.91	nt
Rolipram	0.09	nt

[a]Compounds were tested at five concentrations in the interval 5×10^{-8}–10^{-4} M, in duplicate, and the IC_{50} values were determined by nonlinear regression analysis of inhibition curve using Hill equation curve fitting (Graph Pad Prism, San Diego, CA). [b]nt = not tested.

To prepare compounds **11a–c**, the intermediate (*E*)-3-(3-(cyclopentyloxy)-4-methoxyphenyl)acrylic acid **18** was obtained in high yield by condensing the 3-(cyclopentyloxy)-4-methoxybenzaldehyde **14** with malonic acid in the presence of *N*,*N*-dimethylformamide dimethylacetal (DMF–DMA) and triethylamine, following a recently reported method for carbonyl olefination in mild conditions.[21] The reaction mechanism involves the formation of an enamine intermediate starting from malonic acid and DMF–DMA; the latter reacts with the carbonyl group of compound **14**, giving an iminium intermediate which evolves in the final *trans*-cinnamic acid. The subsequent reaction with the suitable cycloamine in the presence of diphenylphosphorylazide (DPPA) gave the desired **11a–c** as reported in Scheme 2.

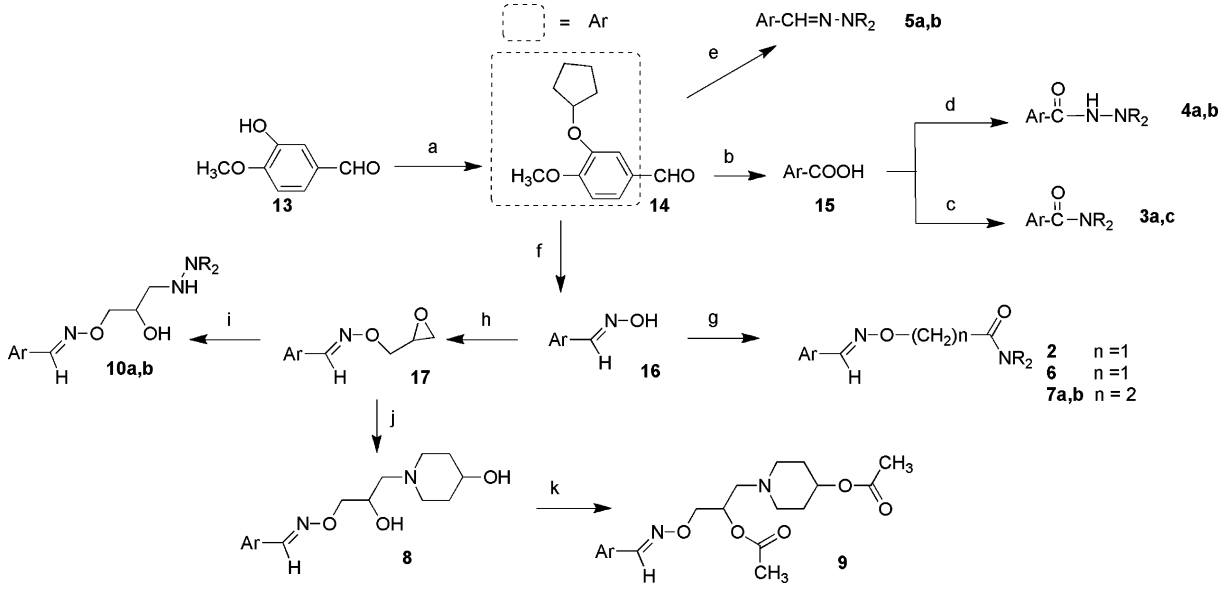
Then, the reduction of (*E*)-3-(3-(cyclopentyloxy)-4-methoxyphenyl)acrylic acid **18** by catalytic hydrogenation gave the corresponding phenylpropyl acid **19**, which was then reacted with the appropriated cycloamine to obtain compounds **12a–c** (Scheme 2).

The building blocks 4-(chloroacetyl)morpholine **20a**, 4-(chloroacetyl)-2,6-dimethyl-morpholine **20b**, and 4-(3-chloropropanoyl)morpholine **20c** have been already reported in the literature,[22–24] but we prepared them by a new method not yet described, which was also used for the synthesis of the 4-(3-chloropropanoyl)-2,6-dimethyl-morpholine **20d**, as described in Scheme 3, whereas, 1-(chloroacetyl)piperidin-4-ol **21** was prepared following a literature method (Scheme 3).[25]

Enzymatic Assays. Preliminary tests on PDE4D3 activity were performed using the recombinant human enzymes expressed in a baculoviral system. Our compounds were tested in duplicate at the concentration of 10 μM, as previously reported.[17] The results are expressed as a percent inhibition of control activity (see Table 1). To obtain information about their selectivity, the most active compounds have been further tested on PDE4A4, PDE4B2, and PDE4C2 (the most representative among the numerous PDE4 splice variants). The results (percent of inhibition at 10 μM concentration) are reported in Table 2. Compounds exerting inhibitory effect higher than 50% were further tested on the same isoform enzyme at five concentrations in the interval 5×10^{-8}–10^{-4} M.

IC_{50} values (μM) for Rolipram, **1**, and **2** (reference compounds) and tested compounds (Table 3) were determined by nonlinear regression analysis of the inhibition curve (see Supporting Information) using Hill equation curve fitting (Graph Pad Prism software). The IC_{50} values obtained for the reference compounds were within accepted limits of historical averages obtained (0.5 log unit).

Scheme 1[a]



[a]Reagents and conditions: (a) bromocyclopentane, K_2CO_3, KI, anhyd DMF, 65 °C, 22 h, yield 83%; (b) sulphamic acid, NaClO, AcOH, 18–20 °C, 1.5 h, yield 91%; (c) $SOCl_2$, 80 °C, 2 h, then, cycloamine, TEA, DCM, 80–60 °C, 6 h, yields 53–67%; (d) $SOCl_2$, 80 °C, 2 h, *N*-aminocycloamine, TEA, DCM, 80–60 °C, 6 h, yields 87–96%; (e) *N*-aminocycloamine, anhyd toluene, reflux, 10 h, yields 71–75%; (f) NH_2OH·HCl, $NaHCO_3$, H_2O/EtOH, rt, 4 h, yields 61%; (g) chloroacetylamines or chloropropanoylamines, anhyd DMF, K_2CO_3, 50–60 °C, yields 44–52%; (h) NaOEt/EtOH, epichlorohydrin, anhyd DMF, 40–50 °C, 12 h, yield 60%; (i) method A, for **10a**, morpholin-4-amine hydrochloride, TEA, absolute ethanol, 40–50 °C, 18 h; yield 28%; method B, for **10b**, 2.6-dimethylmorpholin-4-amine, 50 °C, 18 h, yield 33%; (j) piperidin-4-ol, anhyd THF, 50 °C, 18 h, yield 70%; (k) acetic anhydride, 50 °C, 5 h yield 64%.

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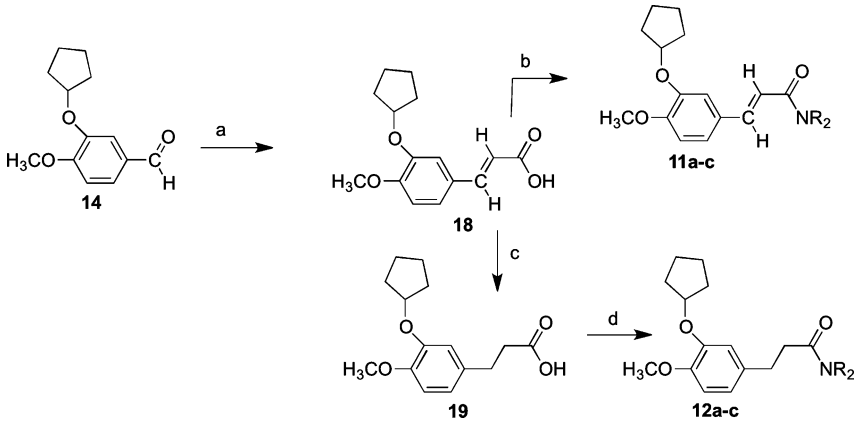
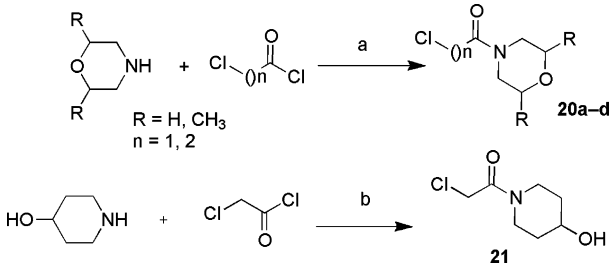
Scheme 2[a]



[a]Reagents and conditions: (a) malonic acid, TEA, DMF−DMA, anhyd toluene, 80 °C, 4 h, yield 91%; (b) cycloamine, anhyd DMF, DPPA, 80 °C, 12 h, yields 47−49%; (c) H_2, 5% Pd/C, methanol, flow 1 mL/min, full-H_2 mode, quantitative yield; (d) cycloamine, anhyd DMF, DPPA, 80 °C, 12 h, yields 44−50%.

Scheme 3[a]



[a]Reagents and conditions: (a) anhyd K_2CO_3, anhyd toluene, 2 h, 60 °C, yields 70−82%; (b) Na_2CO_3 satd solution/ethyl acetate mixture (1:2), rt 2 h, yield 84%.

In Vitro Genotoxic and Cytotoxic Effect Evaluation. To evaluate the preliminary toxic profile of the most interesting compounds in terms of activity and selectivity (**5a**,**b**, **8**, **10a**,**b**), we performed cytotoxicity and genotoxicity assays on human neuronal cells (HTLA).

For the cytotoxic potential, we analyzed the lactate-dehydrogenase release in cells exposed for 24 h to high concentrations (100 μM) of test compounds and **2** (as reference compound) (Table 4).

To evaluate genotoxicity, we analyzed the phosphorylation of the chromatin-bound histone H2AX (γ-H2AX), which is a quantitative marker for the DNA damage response at the site of double-strand breaks.[26] To this purpose, we performed immunoblot analysis on protein extracts from HTLA cells exposed for 24 h to the different compounds. As a positive control, we used etoposide, a topoisomerase II inhibitor that induces DNA double-stranded breaks[27] (Figure 3).

Table 4. Relative Cytotoxic Potential of Compounds 5a, 5b, 8, 10a, 10b, and 2 as Reference Compound[a]

tested compd	cytotoxicity (%)
positive control	100 ± 4.5
DMSO	0.76 ± 0.37
5a	0.04 ± 0.1
5b	0.01 ± 0.61
8	1.2 ± 1.42
10a	0.01 ± 0.62
10b	0.75 ± 1.8
2	0.14 ± 0.18

[a]Data represent the mean ± SEM for three independent experiments.

In Vitro cAMP-Enhancing Potential. To verify the capability of enhancing the accumulation of cAMP, we used a cAMP-specific enzymatic immunoassay (EIA) in HTLA cells treated for 30 min with compound **8** and **2** as a positive control. To increase the basal level of intracellular cAMP, cells received, where indicated, the activator of adenyl cyclase forskolin during the last 20 min of incubation (Figure 4).

Molecular Modeling Studies. All compounds were built, parametrized (Gasteiger−Huckel method) and energy minimized within Sybyl using Tripos force field.[28] Previous studies performed by us[17] did not highlight any striking difference in the inhibitory activity between *E* and *Z* isomers for these derivatives; however, in the preliminary docking studies, for each compound both isomers were considered. The 3D models of PDE4B and PDE4D were extracted from the PDB repository (PDB codes 1XMU and 1XOQ, respectively).[29] Docking simulations were performed using Autodock4.2[30] running under Windows 7 OS (more details have been reported in Supporting Information). Water molecules conserved in all PDE4D structures deposited into PDB were considered for calculations, according to the procedure described by some of us.[17]

DISCUSSION AND CONCLUSIONS

Among the numerous synthesized compounds, **5a**, **5b**, **8**, **10a**, and **10b** resulted in good PDE4D3 inhibitors, **5a** and **10b** being the most active ones, while the inhibition was poor for **3a**, **4a**, and **7a**, very low for **3b**, **3c**, **6**, and **7b**, and practically absent for **4b**, **9**, **11a−c**, and **12a−c**.

Compounds **5a**, **5b**, **8**, **10a**, and **10b** completely inhibited PDE4D3 enzyme at 100 μM concentration and showed a dose−response curve typical of full inhibitors as the previous **1** and **2** (see dose−response curves in Figure 1, Supporting Information).

To support the biological data, we performed a docking study on the tested compounds and the active site of PDE4D, using **1** and **2** as reference compounds (Figure 2A).

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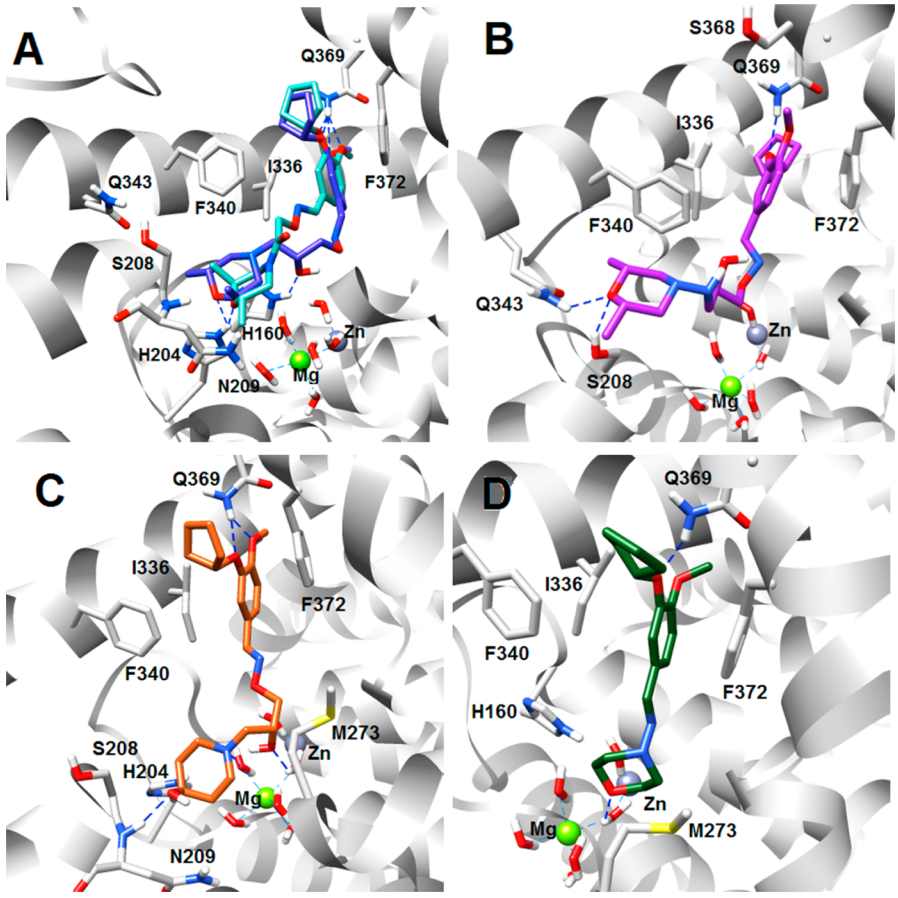

Figure 2. (A) Comparison of the binding of **1** and **2** (C atoms in **1** are colored in purple, in **2** are colored in cyan). (B) Binding mode of **10b** (C atoms are colored in magenta). (C) Binding of **8** (C atoms are colored in orange). (D) Binding of **5a** (C atoms are colored in dark green) into PDE4D. Residues involved in interactions with the inhibitors are reported and colored by the default atom type color. The zinc and magnesium ions are represented as green and purple spheres, respectively, and the coordinate waters are shown as default colored stick. The H-bonds between each ligand and the active site amino acids are shown in blue.

Because the activity data of the synthesized inhibitors are referred to the mixture of the *E* and *Z* isomers, for **8** and **10b**, the more active compounds of this series, we preliminary analyzed the different docking poses of the two geometric isomers. The best solutions of the calculations performed by us (Figure 2, Supporting Information) highlighted two different binding modes inside the active site of the enzyme and only the bonds between the catecholic system and residues Q369 and F372 were shown by both isomers. In fact, **8** and **10b** *E* isomers resulted stretched toward the metal binding pocket, while *Z* isomers showed a folded conformation orienting their scaffold to a mostly hydrophobic area and were unable to extend inside the binding pocket. As a consequence, according to our calculations, *E* isomers could more easily anchoring into the active site than *Z* isomers, thanks to a net of various H-bond with some polar residues located in the metal binding pocket. This observation, in agreement with our previous studies,[17] let us hypothesize for the *Z* isomers a lower inhibitory activity in comparison with the *E* ones. Thus, only these latter were considered in the subsequent docking studies.

The docking studies highlighted a great conformational variability of the inhibitors inside the catalytic site: however, all the minimum energy conformations for the *E* isomer of the active compounds **5a**, **5b**, **8**, **10a**, **10b**, **1**, and **2** share a common binding mode. In fact, they are all anchored by H-bonds between the NH_2 of residue Q369 and the catecholic oxygens of the ligand plus a $\pi-\pi$ stacking between F372 and their catecholic ring. Additional common van der Waals interactions increase the inhibitors stability into the catalytic site and are made by each ligand and residues M273, T333, F340, and I336.

The most active compounds **10a** and **10b** showed IC_{50} values of 1.26 and 0.21 μM, respectively. As depicted in Figure 2B, **10b** binds to the enzyme, making extra H-bonding interactions with two different residues, S208 and Q343, involving the oxygen atom of its morpholine or dimethylmorpholine moiety.

Compound **8** is one of the most potent and selective among the newly synthesized inhibitors having an IC_{50} value of 1.79 μM, about 2 times lower than the reference hit (**1**). In Figure 2C, we reported the docking pose of **8** into the catalytic pocket of PDE4D. As it could be observed, this activity improvement could be justified by the fact that this inhibitor makes three extra H-bonds with two water molecules and the backbone of residue N209, involving the OH group on the 4-hydroxypiperidine moiety, the OH positioned on the linker, and the 4-hydroxypiperidine moiety nitrogen, respectively. Further experimental data confirmed those docking observation. Indeed, compound **9**, being the diacethylester derivative of **8**, was almost inactive on PDE4D3. Our previous investigations showed that the esterification on the hydroxypropyl chain of compound **1** (the dimethyl-morpholine analogue of **8**) did not cause a negative effect on the enzymatic inhibition.[17] Thus, we

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can conclude that the hydroxy substituent on the piperidine moiety is specifically responsible for activity improvement observed for **8**, improvement that does not occur if the hydroxy group is not free.

Among the shorter derivatives, benzamides **3** and amino-benzamides **4** were inactive, while the hydrazones **5a** and **5b**, having the same length of compounds **4**, were very active, with IC_{50} values of 0.66 and 1.55 μM, respectively; in particular, **5a** was about 5 times and 3 times more active than **1** and **2**, respectively, on PDE4D3. The docking simulation for this compound revealed that its shorter chain, in comparison to **3** and **4** (data not shown), is able to provide an H-bond between the morpholine oxygen and a water molecule in the metal pocket of the catalytic site (Figure 2D). This interaction is prevented in **3** and **4**, suggesting a possible rationale for explaining their inactivity.

Also compounds **11** and **12**, having a similar unsaturated or saturated carbon chain, showed a very low inhibitory activity. Indeed, even if the in silico binding mode highlighted an H-bond interaction of the carbonyl group with residue H160, the strong anchorage of the terminal amino group displayed by the most active compounds was lost, with a consequent decrease of the activity. This lack of interaction between the oxygen in the amino terminal function and the enzyme S pocket, seems to be responsible for the activity decrease, as suggested by the experimental data of the inactive compounds **7**, the homologous of **2** (Figure 3, Supporting Information).

However, the **2** analogue (**6**) having a hydroxypiperidine instead of a morpholine in the chain end was completely inactive despite the docking pose showed an H-bond interaction between Q343 and its hydroxy-piperidine group (Figure 3, Supporting Information).

As concerns the ability to discriminate among the different PDE4 isoform, we achieved our goal particularly with compounds **8** that potently inhibited PDE4D3 (IC_{50} = 1.79 μM), being almost inactive on PDE4A4, PDE4B2, and PDE4C2 (inhibition <10%). Also compounds **10a** and **10b** were interesting: in particular, **10a** showed a poor inhibition of PDE4A4, PDE4B2, and PDE4C2 (<40%), while **10b** was almost inactive on PDE4A4 and PDE4C2 and inhibited PDE4B2 with IC_{50} = 18 μM (activity ratio PDE4B2/PDE4D3 = 90). By the selectivity point of view, compound **5a** was less interesting despite its strong activity toward PDE4D3. Indeed, it inhibited also PDE4B2 with IC_{50} = 1.6 μM (activity ratio PDE4B2/PDE4D3 = 2.6).

In 2008, Srivani and co-workers investigated the features of the M-pocket of the catalytic site of both PDE4D and PDE4B to obtain more useful information for a rational design of selective inhibitors.[31] By calculating MOLCAD surface and different physicochemical properties of both PDE4 isoforms with different ligands, they evidenced a higher lipophilicity in the PDE4B M-loop with respect to that of PDE4D. Those observations could be a correct key to understand the selectivity of compounds **8**, **10a**, and **10b** that are the most polar among the new inhibitors here presented.

However, our in silico calculations were not particularly informative on this issue because the comparison between the binding poses of **5a**, **5b**, **8**, **10a**, and **10b** inside PDE4B (data not shown) and PDE4D catalytic sites did not highlight any specific different pattern of interactions.

Thus, the standard docking approach, both on PDE4B and PDE4D, seems able to give only an approximate picture of the ligand−enzyme interaction phenomenon, which is instead very complex and influenced by many different elements. This is why long molecular dynamics simulations are now in progress so as to contribute in a more consistent way to clarify the activity and selectivity of this class of compounds. In any case, only experimental data provided by X-ray studies will be able to clarify this issue.

The results of the lactate-dehydrogenase test clearly indicate that, at least under the tested conditions, neither **5a**, **5b**, **8**, **10a**, **10b**, nor **2** exerted cytotoxic effects (Table 4).

As concerns the genotoxicity test, the etoposide treatment of HTLA cells led to a robust DNA damage, which was not observed by exposing the cells to **5a**, **5b**, **8**, **10a**, **10b**, or **2** (Figure 3).

In addition, in cultured neurons, both **2** and **8** have been able to significantly increase the accumulation of the FSK-induced cAMP without affecting the basal cAMP levels (Figure 4).

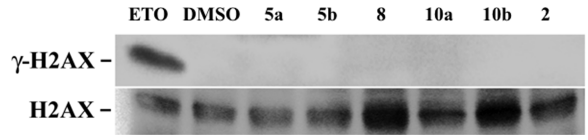

Figure 3. Genotoxic potential. Western blot analysis of γ-H2AX in HTLA cells treated for 24 h with 100 μM etoposide, **5a**, **5b**, **8**, **10a**, **10b**, and **2**, or an equal volume of solvent (DMSO). The H2AX signals represent the internal loading control. The figure is representative of three independent experiments all showing essentially similar results.

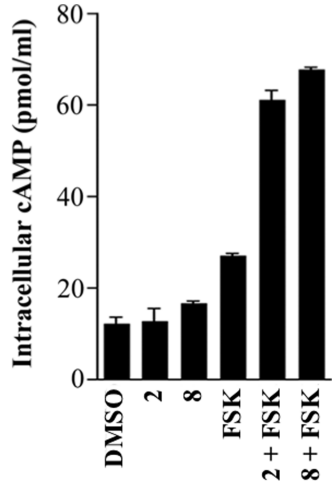

Figure 4. Quantification of intracellular cAMP by specific enzyme immunoassay (EIA). HTLA cells were pretreated for 10 min with **2** (100 μM), compound **8** (100 μM), or an equal volume of DMSO. Then, 1 μM forskolin (FSK) was added, where indicated, for 20 min. At the end of the incubation periods, intracellular cAMP was measured with a cAMP-specific EIA kit according to the manufacturer's instructions. The histogram shows the mean ± SEM for three independent experiments.

In conclusion, the structure modification of our hit compounds **1** and **2** gave new catecholic derivatives able to inhibit PDE4D enzymes with increased potency. The most active compounds showed also a good selectivity for PDE4D3 against PDE4A4, PDE4B2, and PDE4C2 isoforms. In the past few years, different studies reported that selective PDE4D inhibitors have antidepressant activity and cognitive-enhancing effects without causing emesis.[18,32,33] We also reported that

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chronic prophylactic treatment with our selective PDE4D inhibitor **2** can improve memory in an AD mouse model.[19] These results underline that it is demanded to clearly define the molecular features for selective interaction with specific PDE4 isoforms. Differently than the allosteric PDE4D inhibitors reported by Burgin and co-workers,[6] our molecules act directly into the catalytic pocket of the enzyme as the enzymatic profile here reported has confirmed. Unfortunately, the standard docking approach was not completely able to describe the different ligand−enzyme interaction, both on PDE4D and PDE4B, confirming that the phenomenon is very complex and influenced by many different elements. Compound **8**, being the more polar among all the synthesized compounds, resulted in very active and selective inhibitor of PDE4D3 enzyme, supporting the hypothesis of Srivani and co-workers. Compound **8** also enhanced cAMP level in neuronal cells and showed a good preliminary toxicity profile. Further SAR refinements of compound **8** are in progress as well as molecular dynamics simulations to support the rational design of new druggable inhibitors.

EXPERIMENTAL SECTION

Chemistry. Chiminord and Aldrich Chemical, Milan, Italy, purchased all chemicals. Solvents were reagent grade. Unless otherwise stated, all commercial reagents were used without further purification.

Aluminum backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254) were used in thin-layer chromatography (TLC) for routine monitoring the course of reactions. Detection of spots was made by UV light. Merck silica gel, 230−400 mesh, was used for chromatography.

All the amines were purified by distillation before the use. Melting points are not "corrected" and were measured with a Buchi M-560 instrument. IR spectra were recorded with a PerkinElmer 398 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 200 (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. MS spectra were recorded on HP 6890-5973 apparatus (injection temperature 250 °C, HP5 poly(methylphenylsilxane) column 30 m × 0.25 mm, He flux 1 mL/min.

All compounds were tested for purity by TLC (Kieselgel 60F254 DC-Alufolien, E. Merck, Darmstadt, Germany).

Elemental analyses were determined with an elemental analyzer EA 1110 (Fison-Instruments, Milan, Italy), and the purity of all synthesized compounds was >95%.

Hydrogenation was performed with a ThalesNano H-CUBE HC-2.SS, software version: 2.5.0.6.

3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-(2-(2,6-Dimethylmorpholino)-2-oxoethyl) Oxime (2). To a suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde oxime **16** (0.63 g, 2.68 mmol) and anhydrous K_2CO_3 (0.69 g, 5 mmol) in anhydrous DMF (2 mL), 4-(chloroacetyl)-2,6-dimethylmorpholine (**20b**) (1.15 g, 6 mmol) was added. The reaction mixture was stirred at 50−60 °C for 18 h. After cooling to room temperature, the mixture was poured into water (20 mL), the aqueous phase was extracted with diethyl ether (2 × 20 mL), the organic phases were washed with water (2 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The crude product was purified by silica gel (100−200 mesh) column chromatography using a gradient elution [(from diethyl ether alone to a mixture of chloroform/methanol (9:1)] to give a yellow oil. Yield: 76% (lit.: 60%).[17]

General Procedure for 4-[3-(Cyclopentyloxy)-4-methoxybenzoyl]amines (3a−c). To the intermediate 3-(cyclopentyloxy)-4-methoxy-benzoic acid **15** (2.36 g, 10 mmol), previously prepared following the literature procedure,[19] excess thionyl chloride (3.6 mL, 50 mmol) was added, 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 as a crude yellow oil (yield: 1.90 g, 75%), which was used in all the following reactions without further purification.

To a solution of 3-(cyclopentyloxy)-4-methoxybenzoyl chloride (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 mL), anhydrous triethylamine (1.5 mL) and the suitable cycloamine (8 mmol) were added and the reaction mixture was stirred at 60−80 °C for 6 h. After cooling to room temperature, the mixture was washed with 1 N HCl (10 mL), 1 N NaOH (10 mL), and water (20 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The crude was crystallized by addition of a mixture of diethyl ether/petroleum ether (boiling point 50−60 °C) (1:1) yielding white solids (compounds **3a** and **3b**) that were recrystallized from absolute ethanol, or a light-yellow oil (compound **3c**).

*4-[3-(Cyclopentyloxy)-4-methoxybenzoyl]morpholine (**3a**).* Yield 67%; mp 73−74 °C. ^{1}H NMR ($CDCl_3$): δ 1.60−1.83 (m, 8H, 4CH_2 cyclopent), 3.55−3.83 (m, 8H, 4CH_2 morph), 3.89 (s, 3H, OCH_3), 4.75−4.85 (m, 1H, OCH cyclopent), 6.89 (d, J = 8.6 Hz, 1H, H-5 Ar), 6.93−7.08 (m, 2H, H-2 + H-6 Ar). IR (KBr) cm^{-1}: 1637 (C═O). Anal. ($C_{17}H_{23}NO_4$) calcd for C, H, N.

*4-[3-(Cyclopentyloxy)-4-methoxybenzoyl]-2,6-dimethylmorpholine (**3b**).* Yield 53%; mp 91−93 °C. ^{1}H NMR ($CDCl_3$): δ 1.20 (d, J = 6.0 Hz, 6H, 2CH_3 morph), 1.60−2.03 (m, 8H, 4CH_2 cyclopent), 2.50−2.80, 3.20−3.40 and 3.50−3.82 (3m, 6H, 2CH morph + 2CH_2 morph), 3.89 (s, 3H, OCH_3), 4.74−4.85 (m, 1H, OCH cyclopent), 6.88 (d, J = 8.6 Hz, 1H, H-5 Ar), 6.93−7.04 (m, 2H, H-2 + H-6 Ar). IR (KBr) cm^{-1}: 1626 (C═O). Anal. ($C_{19}H_{27}NO_4$) calcd for C, H, N.

*1-[3-(Cyclopentyloxy)-4-methoxybenzoyl]piperidin-4-ol (**3c**).* Yield 92%. ^{1}H NMR ($CDCl_3$): δ 1.31−2.07 (m, 12H, 4CH_2 cyclopent + 2CH_2 pip), 3.02−3.37 (m, 4H, CH_2-N pip), 3.63−4.05 (m, 4H, OCH_3 + *CH*-OH pip), 4.62−4.81 (m, 1H, OCH cyclopent), 6.72−6.99 (m, 3H, H-2 + H-5 + H-6 Ar). IR ($CHCl_3$): cm^{-1}: 3415 (OH), 1671 (C═O). Anal. ($C_{18}H_{25}N_2O_4$) calcd for C, H, N. GC-MS m/z: 319 (M^+).

3-(Cyclopentyloxy)-*N*-(2,6-dimethylmorpholin-4-yl)-4-methoxybenzamide (4b). We applied the same procedure already reported for 3-(cyclopentyloxy)-4-methoxy-*N*-morpholyn-4-ylbenzamide.[15]

To a solution of 3-(cyclopentyloxy)-4-methoxybenzoyl chloride (1.9 g, 7.5 mmol) in CH_2Cl_2 (25 mL), anhydrous triethylamine (1.5 mL) and 2,6-dimethylmorpholin-4-amine (1.04 g, 8 mmol) were added, and the reaction mixture was stirred at 60−80 °C for 6 h. After cooling to room temperature, the mixture was washed with water (20 mL), dried ($MgSO_4$), and concentrated under reduce pressure. The crude was crystallized by addition of a mixture of diethyl ether/petroleum ether (boiling point 50−60 °C) (1:1), yielding a white solid, which was recrystallized from absolute ethanol. Yield 56%; mp 181 °C. ^{1}H NMR ($CDCl_3$): δ 1.21 (d, J = 6.2 Hz, 6H, 2CH_3 morph), 1.50−2.10 (m, 8H, 4CH_2 cyclopent), 2.28−2.48 (m, 2H, CH_2 morph), 3.04−3.22 (m, 2H, CH_2 morph), 3.74−4.20 (m, 5H, OCH_3 + 2CHO morph), 4.72−4.92 (m, 1H, OCH cyclopent), 6.87 (d, J = 8.4 Hz, 1H, H-5 Ar), 6.94 (br s, 1H, NH, disappears with D_2O), 7.20−7.42 (m, 2H, H-2 + H-6 Ar). IR (KBr) cm^{-1}: 3211 (NH), 1634 (C═O). Anal. ($C_{19}H_{28}N_2O_4$) calcd for C, H, N.

***N*-{[3-(Cyclopentyloxy)-4-methoxyphenyl]methylene}-2,6-dimethylmorpholin-4-amine (5b).** We applied the same procedure already reported for *N*-{[3-(cyclopentyloxy)-4-methoxyphenyl]-methylene}morpholin-4-amine.[15]

To a suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **14** (2.2 g, 10 mmol) in anhyd toluene (20 mL), a solution of 2,6-dimethylmorpholin-4-amine (1.30 g,10 mmol) in anhyd toluene (10 mL) was added, and the reaction mixture was refluxed in a Dean−Stark apparatus for 10 h. After cooling to room temperature, the solvent was evaporated under reduced pressure. The crude was purified by silicagel (100−200 mesh) column chromatography using a gradient elution [(from dichloromethane alone to dichloromethane/methanol (9:1)], yielding the pure product as a yellow oil. Yield 75%. ^{1}H NMR ($CDCl_3$): δ 1.21−1.42 (m, 6H, 2CH_3 morph), 1.55−2.05 (m, 8H, 4CH_2 cyclopent), 2.22−2.42 (m, 2H, CH_2 morph), 2.80−

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2.98, 3.18−3.28 and 3.50−3.68 (3m, 2H, CH_2 morph), 3.78−4.03 (m, 4H, OCH_3 + CHO morph), 4.16−4.32 (m, 1H, CHO morph), 4.80−4.95 (m, 1H, OCH cyclopent), 6.85 (d, J = 8.0 Hz, 1H, H-5 Ar), 7.10 (dd, J = 8.0, 1.6 Hz, 1H, H-6 Ar), 7.29 (d, J = 1.6 Hz, 1H, H-2 Ar), 7.58 (s, 1H, CH═N). IR (film) cm^{-1}: 1600 (C═N). Anal. ($C_{19}H_{28}N_2O_3$) calcd for C, H, N.

3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-[2-(4-Hydroxypiperidin-1-yl)-2-oxoethyl]oxime (6). To a suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde oxime **16** (0.63 g, 2.68 mmol) and anhydrous K_2CO_3 (0.69 g, 5 mmol) in anhydrous DMF (2 mL), 1-(chloroacetyl)piperidin-4-ol (**21**) (1.1 g, 6 mmol) was added. The reaction mixture was stirred at 50−60 °C for 18 h. After cooling to room temperature, the mixture was poured into water (20 mL), the aqueous phase was extracted with diethyl ether (2 × 20 mL), the organic phases were washed with water (2 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The crude product was purified by silica gel (100−200 mesh) column chromatography using a gradient elution [(from diethyl ether alone to a mixture of chloroform/methanol (9:1)] to give a yellow oil.

Yield: 48%. ^{1}H NMR ($CDCl_3$): δ 1.40−2.02 (m, 12H, 4CH_2 cyclopent + 2 CH_2 pip), 2.49 (br s, 1H, OH, disappears with D_2O), 3.17−3.35 (m, 2H, CH_2−N), 3.63−4.18 (m, 6H, OCH_3 + *CH*−OH + CH_2N), 4.72−4.91 (m, 3H, CH_2O + OCH cyclopent), 6.83 (d, J = 8.0 Hz, 1H, H-5 Ar), 7.03 (d, J = 8.0 Hz, 1H, H-6 Ar), 7.19 (d, J = 1.8 Hz, 1H, H-2 Ar), 8.11 (s, 1H, CH═N). IR (KBr): cm^{-1}: 3430 (OH), 1671 (C═O). Anal. ($C_{20}H_{28}N_2O_5$) calcd for C, H, N. GC-MS m/z: 376 (M^+).

General Procedure for 3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-[3-(Cycloamin-4-yl)-3-oxopropyl]oximes (7a,b). To a suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde oxime **16** (0.9 g, 3.8 mmol) and anhyd K_2CO_3 (1,1 g, 8 mmol) in anhyd DMF (4 mL), the suitable 3-chloropropanoyl-4-cycloamine **20c,d** (9.6 mmol) dissolved in anhyd DMF (1 mL) was added. The mixture was heated at 100 °C for 24 h. After cooling to room temperature, the mixture was poured into water (50 mL) and extracted with diethyl ether (3 × 20 mL). The organic phases were washed with water (2 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4), and concentrated under reduced pressure.

Compound 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(3-morpholin-4-yl-3-oxopropyl)oxime **7a** crystallized as a white solid by addition of a mixture of diethyl ether/petroleum ether (boiling point 40−60 °C) (1:1).

Compound 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-[3-(2,6-dimethylmorpholin-4-yl)-3-oxopropyl]oxime **7b** was purified by silicagel (100−200 mesh) column chromatography using a gradient elution [(from diethyl ether alone to a mixture of chloroform/methanol (9:1)], yielding a pure yellow oil.

*3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-(3-morpholin-4-yl-3-oxopropyl)oxime (**7a**).* Yield 44%; mp 76−77 °C. ^{1}H NMR ($CDCl_3$): δ 1.60−2.09 (m, 8H, 4CH_2 cyclopent), 2.82 (t, J = 3.2 Hz, 2H, CH_2CO), 3.48−3.69 (m, 8H, 4CH_2 morph), 3.89 (s, 3H, OCH_3), 4.51 (t, J = 3.2 Hz, 2H, CH_2O), 4.81−4.96 (m, 1H, OCH cyclopent), 6.87 (d, J = 8.3 Hz, 1H, H-5 Ar), 7.05 (dd, J = 8.3, 1.6 Hz, 1H, H-6 Ar), 7.22 (d, J = 1.6 Hz, 1H, H-2 Ar), 8.01 (s, 1H, CH═N). IR (KBr) cm^{-1}: 1627 (C═O). Anal. ($C_{20}H_{28}N_2O_5$) calcd for C, H, N.

*3-(Cyclopentyloxy)-4-methoxybenzaldehyde O-[3-(2,6-dimethylmorpholin-4-yl)-3-oxopropyl]oxime (**7b**).* Yellow oil. Yield: 52%. ^{1}H NMR ($CDCl_3$): δ 1.22 (d, J = 6.3 Hz, 6H, 2CH_3 morph), 1.59−2.07 (m, 8H, 4CH_2 cyclopent), 2.28−2.47 (m, 1H, H_A CH_2 morph), 2.64−3.02 (m, 3H, H_B CH_2 morph + CH_2CO), 3.44−3.63 (m, 4H, morph), 3.89 (s, 3H, OCH_3), 4.49 (t, J = 6.8 Hz, 2H, CH_2O), 4.81−4.92 (m, 1H, OCH cyclopent), 6.85 (d, J = 8.3 Hz, 1H, H-5 Ar), 7.06 (dd, J = 8.3, 1.8 Hz, 1H, H-6 Ar), 7.22 (d, J = 1.8 Hz, 1H, H-2 Ar), 8.00 (s, 1H, CH═N). IR ($CHCl_3$) cm^{-1}: 1639 (C═O). Anal. ($C_{22}H_{32}N_2O_5$) calcd for C, H, N.

3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-[2-hydroxy-3-(4-hydroxypiperidin-1-yl)propyl]oxime (8). A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(oxiran-2-ylmethyl)-oxime **17** (1 g, 3.43 mmol) and piperidin-4-ol (0.7 g, 6.92 mmol) in anhydrous THF (4 mL) was heated at 50 °C for 18 h. After cooling to room temperature, diethyl ether (20 mL) was added and the organic phase was washed with water (20 mL), dried ($MgSO_4$), and concentrated under reduced pressure, yielding a light-yellow oil which was purified by silicagel (100−200 mesh) column chromatography using a gradient elution (from diethyl ether to a mixture of diethyl ether/methanol (8:2) to give a light-yellow oil. Yield: 70%. ^{1}H NMR ($CDCl_3$): δ 1.49−2.08 (m, 12H, 4CH_2 cyclopent + 2CH_2 pip), 2.08−2.33 (m, 2H, CH_2-N chain), 2.38−2.61 (m, 4H, CH_2-N pip), 2.65−2.87 (m, 1H, OH disappears with D_2O), 2.87−3.08 (m, 1H, OH disappears with D_2O), 3.61−3.82 (m, 1H, *CH*-OH pip), 3.87 (s, 3H, OCH_3), 4.00−4.27 (m, 3H, CH_2-O + *CH*-OH chain), 4.74−4.92 (m, 1H, OCH cyclopent), 6.84 (d, J = 8.0 Hz, 1H, H-5 Ar), 6.99 (d, J = 8.0 Hz, 1H, H-6 Ar), 7.20 (d, J = 1.0 Hz, 1H, H-2 Ar), 8.05 (s, 1H, CH═N). IR (KBr): cm^{-1}: 3435 (OH). Anal. ($C_{21}H_{32}N_2O_5$) calcd for C, H, N.

1-{2-(Acetyloxy)-3-[({[3-(cyclopentyloxy)-4-methoxyphenyl]-methylene}amino)oxy]propyl}piperidin-4-yl Acetate (9). A suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-[2-hydroxy-3-(4-hydroxypiperidin-1-yl)propyl]oxime **8** (0.64 g, 1.63 mmol) and sodium acetate (0.2 g, 2.4 mmol) in acetic anhydride (5 mL) was heated at 40−50 °C for 5 h. After cooling to room temperature, the mixture was poured into water (100 mL) and extracted with diethyl ether (3 × 10 mL); the organic phases were washed with water (3 × 20 mL) and brine (3 × 20 mL), dried ($MgSO_4$), and concentrated under reduced pressure, yielding an oil which was purified by silicagel (100−200 mesh) column chromatography using as eluent first dichloromethane, then diethyl ether. The pure product was obtained as yellow oil. Yield: 64%. ^{1}H NMR ($CDCl_3$): δ 1.52−2.02 (m, 12H, 4CH_2 cyclopent + 2CH_2 pip), 2.08 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.77−3.20 (m, 5H, 2CH_2N pip + CHO pip), 3.89 (s, 3H, OCH_3), 4.32 (d, J = 4.0 Hz, 2H, CH_2N chain), 4.77−5.03 (m, 3H, CH_2-O + *CH*-OH chain), 5.41−5.60 (m, 1H, OCH cyclopent), 6.86 (d, J = 8.0 Hz, 1H, H-5 Ar), 7.03 (dd, J = 8.0, 1.0 Hz, 1H, H-6 Ar), 7.20 (d, J = 1.0 Hz, 1H, H-2 Ar), 8.02 (s, 1H, CH═N). IR ($CHCl_3$): cm^{-1}: 1740−170 (C═O). Anal. ($C_{25}H_{36}N_2O_7$) calcd for C, H, N.

3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-[2-Hydroxy-3-(morpholin-4-ylamino)propyl]oxime (10a). To a solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(oxiran-2-ylmethyl)-oxime **17** (0.73 g, 2.5 mmol)[15] in absolute ethanol (5 mL), morpholin-4-amine hydrochloride (0.53 g, 3.85 mmol) was added and the mixture was heated at 40−50 °C, then triethylamine (0.73 g, 7.23 mmol) was slowly added and the mixture was heated at 50 °C for further 18 h. After cooling to room temperature, the solvent was removed under reduced pressure and the crude was solved in CH_2Cl_2 (15 mL) and washed with water (3 × 5 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure, yielding a crude light-yellow oil which was purified by silicagel (100−200 mesh) column chromatography using a gradient elution [(from diethyl ether alone to diethyl ether/methanol (9:1)]. The pure oil obtained (0.28 g, 0.71 mmol) was crystallized by addition of a mixture of diethyl ether/petroleum ether (boiling point 40−60 °C) (1:1), yielding a white solid. Yield 28%; mp 78−79 °C. ^{1}H NMR ($CDCl_3$): δ 1.55−2.15 (m, 8H, 4CH_2 cyclopent), 2.65−3.22 (m, 6H, 3CH_2N), 3.68−3.80 and 3.82−4.02 (2m, 7H, OCH_3 + 2CH_2O morph), 4.18−4.39 (m, 3H, OCH_2 + *CH*-OH), 4.78−4.94 (m, 1H, OCH cyclopent), 6.85 (d, J = 8.4 Hz, 1H, H-5 Ar), 7.00 (dd, J = 8.4, 1.8 Hz, 1H, H-6 Ar), 7.19 (d, J = 1.8 Hz, 1H, H-2 Ar), 8.51 (s, 1H, CH=N). IR (KBr) cm^{-1}: 3690 (OH + NH). Anal. ($C_{20}H_{31}N_3O_5$) calcd for C, H, N.

3-(Cyclopentyloxy)-4-methoxybenzaldehyde *O*-{3-[(2,6-Dimethylmorpholin-4-yl)amino]-2-hydroxypropyl}oxime (10b). A mixture of 3-(cyclopentyloxy)-4-methoxybenzaldehyde *O*-(oxiran-2-ylmethyl)oxime **17** (1.5 g, 5.15 mmol) and 2.6-dimethylmorpholin-4-amine (3.9 g, 30 mmol) was heated at 50 °C overnight. After cooling to room temperature, the crude was solved in diethyl ether (20 mL) and the organic phase was washed with water (3 × 20 mL), then extracted with 1 N HCl solution (3 × 20 mL). Subsequently, the acid solution was made alkaline with NaOH and extracted with CH_2Cl_2 (3 × 20 mL). The organic phases were washed with water (20 mL), dried ($MgSO_4$), and concentrated under reduced pressure, yielding brown oil that was purified by distillation in high vacuum. Yield 33%; bp 150

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°C/0.4 mmHg. ^{1}H NMR: δ 1.19−1.40 (m, 6H, 2CH_3 morph), 1.55−1.67 (m, 10H, 4CH_2 cyclopent + CH_2N), 2.81 (br s, 1H, OH, disappears with D_2O), 3.44−3.64 (m, 4H, 2CH_2N), 3.82−3.94 (m, 4H, OCH_3 + 1CHO morph), 4.17−4.27 (m, 4H, OCH_2 + O*CH*−OH + 1CHO morph), 4.73−4.91 (m, 1H, OCH cyclopent), 6.85 (d, J = 8.4 Hz, 1H, H-5 Ar), 7.00 (dd, J = 8.4, 1.8 Hz, 1H, H-6 Ar), 7.21 (d, J = 1.8 Hz, 1H, H-2 Ar), 8.06 (s, 1H, CH═N). IR (KBr) cm^{-1}: 3180−3610 (OH + NH). Anal. ($C_{22}H_{35}N_3O_5$) calcd for C, H, N.

General Procedure for 4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]prop-2-enoyl}-cycloamines (11a−c). To a solution of 3-[3-(cyclopentyloxy)-4-methoxyphenyl]acrylic acid **18** (0.30 g, 1.13 mmol) in anhyd DMF (3 mL), triethylamine (0.17 g, 1.64 mmol), the suitable cycloamine (2.26 mmol), and diphenylphosphorylazide (0.39 g, 1.4 mmol) were slowly added at 0 °C. The mixture was heated at 80 °C for 12 h. After cooling to room temperature, the mixture was poured into ice water (50 mL) and acidified with 1 N HCl solution until to pH = 5. The obtained solid was filtered and recrystallized by absolute ethanol.

*4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]prop-2-enoyl}-morpholine (**11a**).* Yield 48%; mp 98−99 °C. ^{1}H NMR ($CDCl_3$): δ 1.20−2.10 (m, 8H, 4CH_2 cyclopent), 3.60−3.80 (m, 8H, 4CH_2 morph), 3.87 (s, 3H, OCH_3), 4.73−4.97 (m, 1H, OCH cyclopent), 6.65 (d, J_{trans} = 16.0 Hz, 1H, CHCO), 6.80−7.70 (m, 4H, H-2 + H-5 + H-6 Ar + *CH*═CHCO). IR ($CHCl_3$): cm^{-1}: 1644 (C═O). Anal. ($C_{19}H_{25}NO_4$) calcd for C, H, N.

*4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]prop-2-enoyl}-2,6-dimethylmorpholine (**11b**).* Yield 50%; mp 48−50 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (d, J = 6.4 Hz, 6H, 2CH_3 morph), 1.52−2.08 (m, 8H, 4CH_2 cyclopent), 3.22−4.20 (m, 6H, 2CHO + 2CH_2N morph), 3.89 (s, 3H, OCH_3), 4.76−4.92 (m, 1H, OCH cyclopent), 6.66 (d, J_{trans} =16.0 Hz, 1H, CHCO), 6.87 (d, J = 8.2 Hz, 1H, H-5 Ar), 7.14 (d, J = 8.2 Hz, 1H, H-6 Ar), 7.29 (d, J = 2.6 Hz, 1H, H-2 Ar,), 7.64 (d, J_{trans} =16.0 Hz, 1H, *CH*═CHCO). IR ($CHCl_3$): cm^{-1}: 1643 (C═O). Anal. ($C_{21}H_{29}NO_4$) calcd for C, H, N.

*1-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]prop-2-enoyl}-piperidin-4-ol (**11c**).* Yield 49%; mp 78−82 °C. ^{1}H NMR ($CDCl_3$): δ 1.49−2.19 (m, 12H, 4CH_2 cyclopent + 2CH_2 pip), 3.30−3.50 (m, 2H, CH_2N pip), 3.91 (s, 3H, OCH_3), 3.95−4.22 (m, 3H, CH_2N pip + *CH*-OH), 4.78−4.93 (m, 1H, OCH cyclopent), 6.76 (d, J_{trans} =15.2 Hz, 1H, CHCO), 6.88 (d, J = 8.4 Hz, 1H, H-5 Ar), 7.06 (d, J = 1.8 Hz, 1H, H-2 Ar), 7.15 (dd, J = 8.4, 1.8 Hz, 1H, H-6 Ar), 7.64 (d, J_{trans} =15.2 Hz, 1H, *CH*═CHCO). IR ($CHCl_3$): cm^{-1}: 1643 (C═O). Anal. ($C_{20}H_{27}NO_4$) calcd for C, H, N.

General Procedure for 4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]propanoyl}-cycloamines (12a−c). To a solution of 3-[3-(cyclopentyloxy)-4-methoxyphenyl]propanoic acid **19** (0.30 g, 1.13 mmol) in anhydrous DMF (3 mL), triethylamine (0.17 g, 1.64 mmol), the suitable cycloamine (2.26 mmol), and diphenylphosphorylazide (0.39 g, 1.4 mmol) were slowly added at 0 °C. The mixture was heated at 80 °C for 12 h. After cooling to room temperature, the mixture was poured into ice−water (50 mL), neutralized with 1 N HCl solution, and extracted with diethyl ether (3 × 10 mL). The organic phase was washed with $NaHCO_3$ saturated solution (2 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4), and evaporated under reduced pressure. The crude was purified by silica gel (100−200 mesh) column chromatography using a gradient elution [diethyl ether/petroleum ether (bp: 40−60 °C) from (1:1) to (7:3)] to afford a pure product as colorless oil.

*4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]propanoyl}-morpholine (**12a**).* Yield: 50.4%. ^{1}H NMR ($CDCl_3$): δ 1.58−2.18 (m, 8H, 4CH_2 cyclopent), 2.62 (t, J = 8.0 Hz, 2H, CH_2Ar), 2.94 (t, J = 8.0 Hz, 2H, CH_2CO), 3.35−3.80 (m, 8H, 4CH_2 morph), 3.85 (s, 3H, OCH_3), 4.72−4.86 (m, 1H, OCH cyclopent), 6.68−6.86 (m, 3H, H-2 + H-5 + H-6 Ar). IR (film) cm^{-1}: 1644 (C═O). Anal. ($C_{19}H_{27}NO_4$) calcd for C, H, N.

*4-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]propanoyl}-2,6-dimethylmorpholine (**12b**).* Yield: 44.3%. ^{1}H NMR ($CDCl_3$): δ 1.19 (d, J = 6.0 Hz, 6H, 2CH_3), 1.57−2.08 (m, 8H, 4CH_2 cyclopent), 2.62 (t, J = 8.0 Hz, 2H, CH_2Ar), 2.94 (t, J = 8.0 Hz, 2H, CH_2CO), 3.35−3.80 (m, 6H, 2CH_2N + 2 CHO morph), 3.85 (s, 3H, OCH_3), 4.72−4.86 (m, 1H, OCH cyclopent), 6.68−6.86 (m, 3H, H-2 + H-5 + H-6 Ar). IR (film) cm^{-1}: 1645 (C═O). Anal. ($C_{21}H_{31}NO_4$) calcd for C, H, N.

*1-{3-[3-(Cyclopentyloxy)-4-methoxyphenyl]propanoyl}piperidin-4-ol (**12c**).* Yield: 48%. ^{1}H NMR ($CDCl_3$): δ 1.33−2.20 (m, 12H, 4CH_2 cyclopent + 2CH_2 pip), 2.65 (t, J = 8.0 Hz, 2H, CH_2Ar), 2.92 (t, J = 8.0 Hz, 2H, CH_2CO), 3.12−3.32 (m, 2H, CH_2N pip), 3.84 (s, 3H, OCH_3), 3.88−4.17 (m, 3H, CH_2N pip + *CH*-OH), 4.72−4.91 (m, 1H, OCH cyclopent), 6.68−6.88 (m, 3H, H-2+ H-5 + H-6 Ar). IR (film) cm^{-1}: 3399 (OH), 1621 (C═O). Anal. ($C_{20}H_{29}NO_4$) calcd for C, H, N.

3-[3-(Cyclopentyloxy)-4-methoxyphenyl]acrylic Acid (18). To a suspension of 3-(cyclopentyloxy)-4-methoxybenzaldehyde **14** (0.44 g, 2 mmol) and malonic acid (0.83 g, 8 mmol) in anhyd toluene (10 mL), triethylamine (1.01 g, 10 mmol) and dimethylformamide dimethyl acetal (0.36 g, 3 mmol) were added. The mixture was refluxed for 4 h. After cooling to room temperature, the solvent was evaporated under reduced pressure and the crude was solved in dichloromethane (20 mL) and the organic phase was extracted with $NaHCO_3$ saturated solution (2 × 10 mL) and then with a 1 N NaOH solution (1 × 10 mL) The aqueous phases were acidified with 1 N HCl solution, and the obtained yellow solid was filtered and washed with water. Yield 90%; mp 194−195 °C (lit. 191 °C).[34]

3-[3-(Cyclopentyloxy)-4-methoxyphenyl]propanoic Acid (19). A solution of 3-[3-(cyclopentyloxy)-4-methoxyphenyl]acrylic acid **18** (0.30 g, 1.13 mmol) in methanol (12 mL) was reduced with H-CUBE apparatus at room temperature in full H_2 mode (flow = 1 mL/min) using 5% Pd/C as catalyst. The obtained solution was concentrated under reduced pressure to afford the product as white solid. Yield 100%; mp 123−125 °C (lit. 114−116 °C).[35]

General Procedure for Chloroacetylamines and Cloropropanoylamines (20a−d). To a solution of the suitable cycloamine (23 mmol) in anhyd toluene (60 mL), anhyd K_2CO_3 (6.3 g, 45 mmol) and 2-chloroacetyl chloride or 3-chloropropanoyl chloride (23 mmol) were added; the mixture was heated at 60 °C for 2 h. After cooling to room temperature, the solids (K_2CO_3 and KCl) were filtered off and the solution was concentrated under reduced pressure, obtaining crude oils which were purified by high vacuum distillation to afford final pure compounds as light-yellow oils.

*4-(Chloroacetyl)morpholine (**20a**).* Yield 72% (lit. 17−100%); bp 110−120 °C/0.6 mmHg.[22]

*4-(Chloroacetyl)-2,6-dimethylilmorpholine (**20b**).* Yield 82% (lit. 48%); bp 110−120 °C/0.6 mmHg.[23]

*4-(3-Chloropropanoyl)morpholine (**20c**).* Yield 70% (lit. 85%); bp 110 °C/0.6 mmHg.[24]

*4-(3-Chloropropanoyl)-2,6-dimethylmorpholine (**20d**).* Yield 80%; bp 118 °C/0.6 mmHg.

1-(Chloroacetyl)piperidin-4-ol (21). Piperidin-1-ol (1 g, 10 mmol) was solved in a mixture of saturated Na_2CO_3 solution (75 mL) and ethyl acetate (150 mL), and chloroacetyl chloride (1.2 mL, 15 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. After separation in a glass funnel, the organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to afford pure light-yellow oil that was used without further purification. Yield: 1.18 g, 66% (lit. 84%).[25]

Cytotoxicity and Genotoxicity Assays. The cells used in this study (human neuroblastoma cell line HTLA-230, HTLA) were grown in Roswell Park Memorial Institute medium (RPMI), with 0.1 mM nonessential amino acids and 10% fetal bovine serum.

For the cytotoxicity assay, cells were treated for 24 h with 100 μM of the indicated compounds dissolved in DMSO. Control cells received the same volume of solvent (1 μL/mL medium). At the end the incubation period, conditioned media were analyzed for lactate-dehydrogenase release using the Cytoxicity Detection Kit^{PLUS} (Roche, Germany) according to manufacturer protocols.

To evaluate genotoxicity, HTLA cells were treated for 24 h with the indicated compounds dissolved in DMSO and then processed for total protein extraction as described previously.[36] Immunoblots were done according to standard methods, using the following antibodies: mouse monoclonal [2F3] to γ H2A.X (phospho S139) and rabbit polyclonal to Histone H2A.X (Abcam, UK); antimouse and antirabbit secondary

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antibodies coupled to horseradish peroxidase (GE Healthcare, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's instructions (GE Healthcare). Chemiluminescence was monitored by exposure to films, and signals were analyzed under nonsaturating condition with an image densitometer (Bio-Rad, CA, USA).

cAMP Enzymatic Immunoassay (EIA). Quantification of intracellular cAMP was performed with DetectX Direct Cyclic AMP Enzyme Immunoassay Kit (Arbor Assay, MI, USA), following the manufacturer's protocol. cAMP levels were calculated according to the standard curves prepared on the same EIA plates.

■ ASSOCIATED CONTENT

Supporting Information

Dose response curves for most active compounds **5a**, **5b**, **8**, **10a**, **10b**, and hit compounds **1** and **2**; binding mode of compounds **6**, **7b**, and **11b** into PDE4D; comparison of the binding of the two geometric isomers of **8** and **10b** into PDE4D; elemental analyses of compounds **3**–**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AD, Alzheimer disease; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; cGMP, cyclic guanosine monophosphate; hydrogen bond acceptor (HBA); hydrogen bond donor (HBD); PDE, phosphodiesterase; PDE4Is, PDE4 inhibitors; SAR, structure–activity- relationship

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