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Original article

CODES, a novel procedure for ligand-based virtual screening: PDE7 inhibitors as an application example

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Abstract

Phosphodiesterase (PDE) 7 is a high affinity cAMP-specific PDE whose functional role in T-cells has been the subject of some controversy. Recent findings on tissue distribution, however, support the hypothesis that PDE7 could be a good target for the treatment of airway diseases, T-cell related diseases or central nervous system (CNS) disorders. Therefore, the identification of selective inhibitors targeted against PDE7 enzyme has become an attractive area of research. We report here the first use of the descriptors generated by the CODES program for ligand-based virtual screening. This program codifies molecules from a topological point of view and the generated descriptors are related to the chemical nature of the atoms, the atomic bonds and the connectivity with the rest of the molecule. They are also able to distinguish among stereoisomers. By using this approach, 173 compounds were codified, and their similarity with the reference compound was analysed. Based on the analysis, new potential PDE7 inhibitors have been identified, synthesized and biologically evaluated confirming that CODES descriptors are valid for ligand-based virtual screening and provided new lead compounds for further optimization as potent and selective PDE7 inhibitors.

Keywords: PDE7; Virtual screening; CODES

1. Introduction

Phosphodiesterases (PDEs) comprise a large family of metallophosphohydrolases enzymes that metabolize the ubiquitous second messengers adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) to their respective inactive 5′-monophosphates [1]. cAMP and cGMP are generated through the action of adenylyl cyclase and guanylyl cyclase, respectively, and serve to transduce the action of many hormones, neurotransmitters, and other cellular effectors [2].

One mechanism by which cAMP/cGMP may be elevated within cells is by inhibition of cyclic nucleotide PDEs, which are the only way to degrade them [3]. Based on the fact that agents with the ability to elevate intracellular cAMP levels have been demonstrated to possess immunosuppressive and anti-inflammatory properties [4], the interest on the development of specific PDEs inhibitors and their role on immunomodulation processes have been renewed [5]. Thus, selective inhibitors of cAMP-specific PDEs have been suggested as therapies for the treatment of several human diseases [6], predominantly immunological disorders, such as multiple sclerosis [7], and inflammatory systems [8] and also disorders of the CNS such as depression, ischemia–reperfusion injury, and Alzheimer disease [9–12].

From the large phosphodiesterases family, PDEs isoenzymes 3B, 4A, 4B, 4D and 7A1 are predominant in immune cells [13–15]. To date, most of the research has been centered on PDE4 inhibitors because PDE4 represents a major

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isoenzyme in most T-cell preparations and their selective inhibitors are able to reduce inflammatory cytokines production [15,16]. However, a major drawback of these compounds has been the significant side effect of emesis [17]. To avoid these adverse effects several strategies have been considered to dissociate the beneficial and detrimental effects of PDE4 inhibitors with some degree of success and the second generation of PDE4 inhibitors has shown better pharmacokinetics profiles and less side effects [18–20].

An alternative approach is to target other cAMP–PDE families that are expressed in proinflammatory and immune cells. Initial evidence indicated that PDE7 had an important role in the activation of T-cells [21,22], however, results based on the use of PDE7A (−/−) knock out mice failed to confirm the role of PDE7A in T-cell proliferation and suggested that this phosphodiesterase could have some other role in the regulation of humoral immune responses [23]. Thus, the use of selective PDE7A inhibitors will be pivotal to elucidate the true potential of PDE7A as a pharmacological target in the context of the immune response [24]. Several years ago, our research group was the first one in reported the first PDE7 selective inhibitors [25]. Since then, a lot of efforts have been done to increase potency and selectivity of this kind of compounds [26], conforming a great variety of diverse chemical compounds with interesting pharmacological profiles [27].

The search for new lead compounds in the pharmaceutical industry increasingly makes use of virtual screening of databases for drug discovery. Among the available methods, similarity searching is a cheap and widely used method to distill a pool of potentially interesting compounds from a large database [28]. This method is based on the similarity principle, one of the crucial principles of the rational drug design, that states that structurally similar compounds are expected to have similar physicochemical and biological properties, and therefore, they could have similar in vivo effects. Thus, once an active molecule has been identified, the ligand virtual screening is based in the search in codified databases of similar structures regarding similarity or any other specific property. This has two main advantages: a high speed in comparison to the direct analysis of each molecule in a database, and the ability to find molecules that at a first glance do look similar but, however, they hide the same features that have defined the target molecule [29].

In the present study, we introduce CODES as a novel procedure for ligand-based virtual screening. This program, which generates topological descriptors based on calculations with neural networks (NN), has never been used for virtual screening purposes, although it has been widely used for QSAR studies, as for example the prediction of the nematocide action of pteridine derivatives [30], as well as new potassium channels openers [31]. In this way, it has also been used to carry out QSPR studies as the determination of pharmacokinetic properties as for example the oral absorption and BBB penetration of several drugs [32] and the mean life of antihistaminic drugs, classifying them in their corresponding therapeutic categories [33].

To evaluate the efficiency of such method, we conducted a virtual screening focused on the finding of new phosphodiesterase (PDE) 7 inhibitors. After the identification of lead compounds, we synthesized new derivatives based on the results of such screening and determined their inhibition on PDE7.

2. Materials and methods

Similarity searching begins with the identification of a known bioactive molecule, the target structure. The structure of this molecule is then compared with each of the structures in a database using an objective measure of similarity. The target molecule and the database may comprise 2D and 3D structures, which are characterized by one or more descriptors that describe some structural features of the molecules under scrutiny [28]. Later on a similarity coefficient is used to quantify the degree of resemblance between the target structure and each of the database structures. The database molecules are then ranked in decreasing order of the calculated similarity values, with the top-ranked molecules, being those that bear the closest structural similarity to the target structure [34].

The effectiveness of the search depends on both the representation and similarity coefficient used, which together comprise the overall similarity measure.

2.1. Selection of the target molecule and the database

In the search of new PDE7 inhibitors, we selected as target molecule the previously published 1-[(3,4-dichlorophenyl)-methyl]-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide (Fig. 1), because it was the first PDE7 inhibitor described with an IC_{50} value of 8 μM [25].

The database was created with a set of 173 structurally diverse molecules (**1–173**). This collection of compounds included 131 structurally diverse derivatives (**1–131**) selected because they have a biologically privileged scaffold that show different pharmacological and toxicological properties, but it was also enriched with 31 PDE7 inhibitors (**132–162**), including the target structure as **134**, and 11 PDE4 inhibitors (**163–173**) using in different biological assays. (All these structures are included in Supplementary data.)

2.2. Codification of structures: CODES

Databases in which the virtual screening is carried out have their structures codified by descriptors, that is, indexes

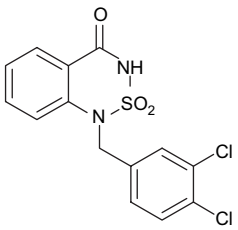


Fig. 1. Target structure for the virtual screening of new PDE7 inhibitors.

calculated on the basis of one or several properties of those structures that possess essential information about the nature and/or structure of those molecules. Descriptors can be derived from 1-, 2- or 3-dimensional representations of the molecule [29].

In this work the structures have been codified using CODES program. These descriptors are related to the chemical nature of the atoms, the atomic bonds and the connectivity with the rest of the molecule. Moreover, apart of all the information of the chemical structure, they show two additional advantages over other topological descriptors: they are able to distinguish among stereoisomers and, at the same time, avoid all the problems associated with the 3D molecular conformation. As it is described in section theoretical basis of CODES of Supplementary data, the output of this program is a matrix in which each of the columns represents one of the atoms of the molecule.

2.3. Reduction of dimensions

In general, most similarity measures depend on dimensionality [35], mean that they can only compare two structures with the same dimensions, that is, with the same number of descriptors. In the case of CODES, the matrix depends on the number of atoms of the molecule. Therefore, once all the molecules have been codified, it is necessary to reduce the dimensions to homogenize them in all the data set, that is, to be able to compare all the molecules independently of their nature. A widely spread approach is to reduce the dimensionality of the original descriptors by means of principal component analysis (PCA), selecting only the few first most relevant principal components. Components of this new variable have to be a linear combination of the original descriptors and they must explain a proportion of the variance of the data big enough [36]. Therefore, it summarized all the available information with the minimum loss of explaining capacity with respect to the variance.

In the case of CODES' matrixes, the reduction of dimensions by PCA was done by Tsar (version 3.3. Oxford Molecular, Ltd.), finding that the two first components were able to explain 95% of the variance and 99% of the times. That is why we select these two variables to define each compound of the database.

2.4. Similarity criteria

For the final comparison of all the structures of the database a similarity criteria needs to be used. Among all the similarity measures between continuous topological descriptors, Tanimoto coefficient was selected in this work because it has been shown to be the most effective one in the identification of active compounds from a target molecule [34]. Anyway, this measure is monotonic with the Dice coefficient, is highly related with the Cosine coefficient, and for binary descriptors its complement is coincident with the Soergel coefficient. So, any of these measures provides a good indication of similarity [28].

3. Results and discussion

Results were ordered in a decreasing way depending on their similarity with the target molecule (Table 1). Results for those molecules whose coefficient was higher than the threshold of 0.9 and of 0.95 are shown in Fig. 2. We only select those molecules because the capacity to find new active compounds increases with the selected threshold [37]. As it can be observed, the only molecule with Tanimoto coefficient equal to 1 is **134**, because it was the target molecule.

From a first analysis of these structures it is possible to observe that only 11 out of 173 molecules (6.3%) have a Tanimoto coefficient higher than 0.95, and only 17 (9.8%) have it higher than 0.90. Therefore this can be considered as a selective and efficient method for virtual screening by similarity. Moreover molecules **136**, **137**, **135** and **132** belong to the benzothienothiadiazine family, which is structurally similar to the target molecule; **146** and **152** are PDE7 inhibitors, what indicate that this process retrieves potentially active molecules against a biological target; and finally the most interesting result falls on the structures **173**, **164** and **163**, because they are PDE4 inhibitors, enzyme with a high structural homology with PDE7.

On the basis of these results, we propose the synthesis not only of new thiadiazine derivatives analogues to the target molecule (**174**–**180**), but also quinazoline derivatives (**181**–**196**), structurally analogues to nitraquazone (**173**) (Scheme 1).

For the preparation of the newly designed benzothiadiazine derivatives, we alkylated the corresponding heterocycles according to previously described procedures [25,38]. On the other hand, the quinazoline derivatives proposed were prepared following the procedures previously described [39–46] by cyclocondesation of the corresponding functionalised heterocycles with isocyanates or isothiocyanates.

These novel sets of compounds were tested for their inhibitory potencies against human recombinant PDE7 expressed in *Saccharomyces cerevisiae* as described in Section 5. In this expression system the only cyclic nucleotide hydrolysing activity present in cell extracts corresponded to human PDE7. Isoenzyme selectivity was obtained by comparing the IC_{50} values or % of inhibition of the compounds against PDE7 with their inhibitory activity against PDE4 and PDE3 (Table 2).

Some of the heterocyclic compounds evaluated exhibited PDE7 inhibitory properties (IC_{50} at μM level) validating the lead compounds identified by similarity virtual screening. From the biological results, preliminary structure-activity relationships have emerged. Regarding the heterocyclic system, the thioxoquinazoline ring appears more effective than the other condensed heterocycles (**185**, **193** and **195** versus **175** and **179**). Moreover, derivatives with a non-substituted phenyl (**183** and **192**) or *ortho*-halogen are the most active ones of each series (**185** and **187**).

4. Conclusions

We have shown the utility of CODES neural networks molecular codification as a useful tool for ligand-based virtual

screening of compounds within a wide range of chemical diverse structures and have been efficiently applied to identify new PDE7 inhibitors. A database of 173 no-congeneric compounds has been codified by descriptors generated by CODES program, the dimensionality of the original descriptors was done by PCA and Tanimoto coefficient was selected as similarity criteria. Final results of virtual screening provided new leads based on thiadiazine and quinazoline scaffolds. Several derivatives structurally related to these leads were synthesized and evaluated enzymatically. The biological data revealed that these novel compounds are equipotent to the target structure but with a simple chemical structure. For this reason, these new compounds may be considered as new prototypes for further optimization. CODES program has been validated for ligand-based virtual screening, proving to be a selective and efficient method for the selection of new PDE7 inhibitors.

5. Experimental

5.1. Chemistry

Melting points were determined with a Reichert–Jung Thermovar apparatus and are uncorrected. Flash column chromatography was carried out at medium pressure using silica gel (E. Merck, Grade 60, particle size 0.040–0.063 mm, 230–240 mesh ASTM) with the indicated solvent as eluent. ^{1}H NMR spectra were obtained on Varian XL-300 and Bruker WP-300 spectrometers working at 300 MHz. Typical spectral parameters were: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K. NOE difference spectra were measured under the same conditions, using a presaturation time of 3 s. ^{13}C NMR experiments were carried out on Varian XL-300 and Bruker WP-300 spectrometers operating at 75 MHz. The

Table 1
Virtual screening results ordered by the Tanimoto coefficient

Entry	Compound	Tanimoto coefficient	Entry	Compound	Tanimoto coefficient	Entry	Compound	Tanimoto coefficient	Entry	Compound	Tanimoto coefficient
1	**134**	1	44	**91**	0.04707651	87	**139**	−0.33141429	131	**82**	−0.33281374
2	**74**	0.99760222	45	**63**	0.03985446	88	**34**	−0.33188710	132	**93**	−0.33282288
3	**39**	0.99686219	46	**22**	0.03355749	89	**107**	−0.33194858	133	**8**	−0.33283736
4	**173**	0.99518338	47	**6**	0.03342122	90	**88**	−0.33196216	134	**86**	−0.33284900
5	**130**	0.98935455	48	**69**	0.03325900	91	**49**	−0.33197592	135	**117**	−0.33285026
6	**98**	0.98190982	49	**81**	0.02953837	92	**44**	−0.33208948	136	**92**	−0.33285890
7	**85**	0.98030514	50	**43**	0.02821337	93	**50**	−0.33212217	137	**168**	−0.33286646
8	**136**	0.97672433	51	**142**	0.02419064	94	**30**	−0.33216254	138	**115**	−0.33286934
9	**120**	0.97370866	52	**121**	0.02066309	95	**94**	−0.33218093	139	**106**	−0.33288291
10	**146**	0.95967932	53	**56**	0.01809397	96	**125**	−0.33227110	140	**13**	−0.33289077
11	**137**	0.95421559	54	**131**	0.01642970	97	**14**	−0.33232989	141	**35**	−0.33289549
12	**59**	0.94632901	55	**3**	0.01586728	98	**52**	−0.33246317	142	**47**	−0.33290870
13	**135**	0.94273906	56	**95**	0.01488014	99	**55**	−0.33247253	143	**18**	−0.33292077
14	**163**	0.93849906	57	**141**	0.01329916	100	**9**	−0.33248705	144	**57**	−0.33293108
15	**152**	0.91761092	58	**96**	0.01130440	101	**40**	−0.33249802	145	**118**	−0.33293197
16	**67**	0.90380532	59	**33**	0.00802879	102	**73**	−0.33252201	146	**36**	−0.33293548
17	**164**	0.90319139	60	**116**	0.00758128	103	**99**	−0.33253986	147	**58**	−0.33293684
18	**132**	0.90238568	61	**60**	0.00667694	104	**78**	−0.33258125	148	**166**	−0.33295283
19	**65**	0.89445047	62	**128**	0.00588713	105	**84**	−0.33258309	149	**101**	−0.33296629
20	**79**	0.88085173	63	**129**	0.00539618	106	**29**	−0.33259591	150	**53**	−0.33298047
21	**161**	0.85530084	64	**10**	0.00470288	107	**124**	−0.33263054	151	**87**	−0.33298630
22	**7**	0.85049195	65	**167**	−0.00459139	108	**162**	−0.33263889	152	**19**	−0.33299286
23	**31**	0.85010393	66	**71**	−0.00871085	109	**42**	−0.33264401	153	**38**	−0.33299979
24	**16**	0.80705312	67	**144**	−0.01016302	110	**145**	−0.33265572	154	**109**	−0.33301542
25	**150**	0.80670073	68	**154**	−0.01708360	111	**90**	−0.33265593	155	**37**	−0.33302045
26	**153**	0.80465623	69	**27**	−0.02322586	112	**122**	−0.33267046	156	**114**	−0.33302122
27	**51**	0.79510746	70	**76**	−0.03710607	113	**75**	−0.33268213	157	**105**	−0.33302414
28	**26**	0.79455399	71	**138**	−0.04287797	114	**111**	−0.33269321	158	**149**	−0.33302877
29	**160**	0.79168535	72	**104**	−0.04553995	115	**23**	−0.33269378	159	**2**	−0.33305239
30	**54**	0.77674841	73	**25**	−0.05763491	116	**62**	−0.33269675	160	**148**	−0.33306977
31	**64**	0.75853641	74	**17**	−0.05789004	117	**61**	−0.33269874	161	**21**	−0.33308175
32	**156**	0.74918709	75	**4**	−0.06634058	118	**65**	−0.33269892	162	**97**	−0.33309358
33	**171**	0.74918663	76	**110**	−0.06888479	119	**147**	−0.33270931	163	**140**	−0.33310867
34	**A28**	0.69955841	77	**170**	−0.07006056	120	**119**	−0.33271742	164	**127**	−0.33312856
35	**113**	0.68273209	78	**48**	−0.07416544	121	**80**	−0.33272102	165	**143**	−0.33313077
36	**158**	0.65609348	79	**162**	−0.07856270	123	**15**	−0.33272778	166	**89**	−0.33313179
37	**155**	0.61968205	80	**126**	−0.08798144	124	**11**	−0.33273716	167	**12**	−0.33316889
38	**157**	0.54941936	81	**159**	−0.08915842	125	**83**	−0.33274624	168	**102**	−0.33317586
39	**153**	0.53978515	82	**41**	−0.09353445	126	**169**	−0.33278687	169	**151**	−0.33318839
40	**100**	0.27538400	83	**108**	−0.19490619	127	**72**	−0.33279869	170	**70**	−0.33327559
41	**103**	0.25228953	84	**124**	−0.19490769	128	**32**	−0.33280073	171	**68**	−0.33328733
42	**45**	0.18791646	85	**46**	−0.19872345	129	**165**	−0.33280428	172	**20**	−0.33330564
43	**5**	0.07573717	86	**1**	−0.32664176	130	**24**	−0.33280748	173	**77**	−0.33332744

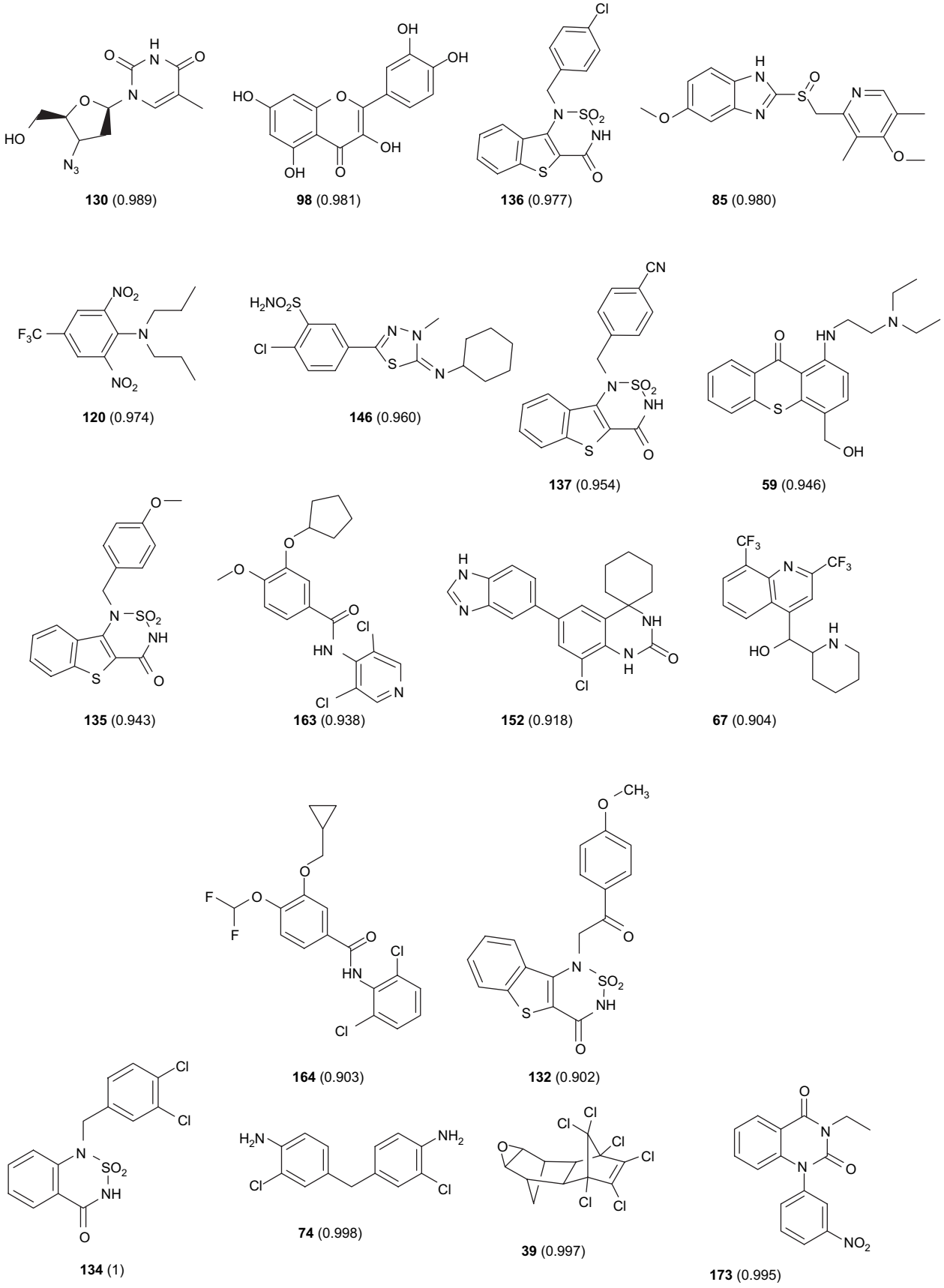
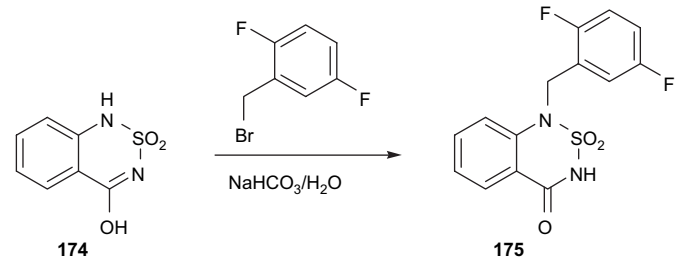
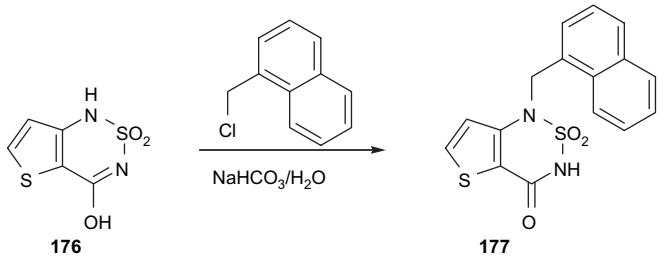


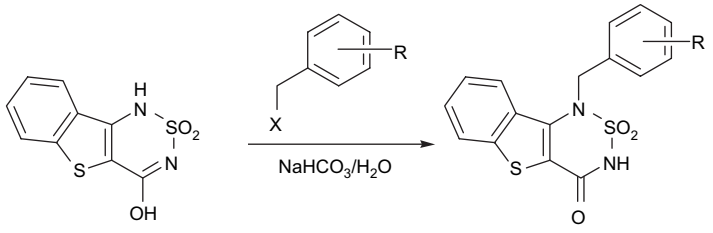
Fig. 2. Structures with Tanimoto coefficient higher than 0.90 (in brackets) obtained virtual screening by similarity with CODES.



174 **175**

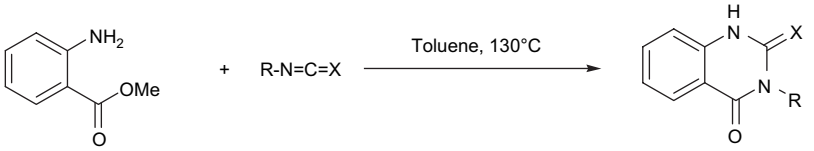


176 **177**

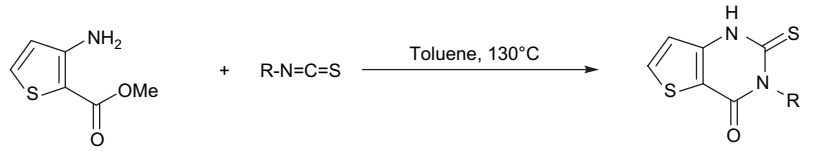


178

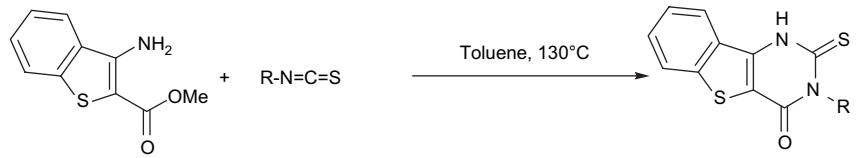
R=2,5-diF **179**
R=3,4-diCl **180**



X = O R=H **181**
X = O R=4-OMePh **182**
X = S R=Ph **183**
X = S R=4-OMePh **184**
X = S R=2,6-diFPh **185**
X = S R=2,3,4-triFPh **186**
X = S R=2-BrPh **187**
X = S R=Bn **188**
X = S R=Naphthyl **189**
X = S R=Me **190**



R = Me **191**
R = Ph **192**
R = 2,6-diFPh **193**



R = Ph **194**
R = 2,6-diFPh **195**
R = 2,3,4-triFPh **196**

Scheme 1.

Table 2
Biological activity (PDE7A, PDE3A, and PDE4B inhibitions) of thiadiazine and quinazoline derivatives **174–196**[a]

Compound	PDE3A (%)	PDE4B (%)	PDE7A (%)
174	0	0	12
175	14.1	12.2	4.1
176	0	1	0
177	82.5	7	26
178	0	3	16
179	30	13.2	3.6
180	12	26.2	25.1
181	59	1.7	8.4
182	4.4	4.4	0.2
183	3.3	21.7	5.5
184	1.7	6.3	2.4
185	6.5	37	5.5
186	2.5	13	48.5
187	2.7	56	1.9
188	8.8	16.4	5.4
189	1.9	5.9	2.1
190	20	12	7
191	30.5	8.5	25
192	8	10	24
193	5	10	41.5
194	7	8.5	11
195	15.5	20.5	66.5
196	4.5	11.5	10

[a] The inhibitory potency of the synthesized compounds on the human PDE3A, PDE4B and PDE7A activities was tested as described in Section 5. Data are indicated as IC_{50} (μM) or percent inhibition at 10 μM ($n = 2–3$).

acquisition parameters were: spectral width 16 kHz, acquisition time 0.99 s, pulse width 9 μs (57°), data size 32 K. Chemical shifts are reported in δ values (ppm) relative to internal Me_4Si, and J values are reported in Hertz. Analytical HPLC was carried out on a Waters 6000 system using a symmetry C18 (5 mm, 100 Å). Isocratic conditions were used: mobile phase CH_3CN/H_2O (0.05% H_3PO_4 + 0.04% Et_3N); flow rate 1 mL/min; detection, UV (254 nm). Elemental analyses were performed by the analytical department at C.N.Q.O. (CSIC) and the results obtained were within ±0.4% of the theoretical values.

*5.1.1. 1-[(2,5-Difluorophenyl)methyl]-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide (**175**)*

To a solution of benzothiadiazine dioxide **174** [47] (0.210 g, 1 mmol) in aqueous saturated solution of sodium bicarbonate (20 mL), 2,5-difluorophenylmethyl bromide (0.310 g, 1.5 mmol) was added. The reaction mixture was refluxed for 3 h. After cooling to room temperature, the aqueous phase was washed with CH_2Cl_2 (1 × 10 mL). The aqueous phase was cooled at −4 °C and the product was isolated by filtration of aqueous phase. Purification: preparative thin layer chromatography, using CH_2Cl_2:MeOH (10:1) as eluent; yield 0.260 g (83%) as a white solid; mp 131–132 °C. Purity 86% (by HPLC); ^{1}H NMR (CD_3OD) δ 5.16 (s, 2H, N–CH2), 6.71 (dd, 1H, $J_{H6H8} = 1.2$ Hz, $J_{H7H8} = 8.3$ Hz, H-8), 6.89 (t, 1H, $J_{H6H7} = 7.3$ Hz, H-6), 7.19 (t, 1H, $J_{H5H7} = 1.7$ Hz, H-7), 7.06–7.99 (m, 3H, Ar–H), 7.90 (dd, 1H, $J_{H5H6} = 7.8$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 42.0 (CH2), 113.4 (C-8), 119.1 (C-6), 119.4 (C-4a), 116.1, 117.2, 118.9, 134.2, 155.6, 157.3 (Ar–C), 128.7 (C-5), 132.1 (C-7), 142.1 (C-8a), 165.6 (C-4). Anal. $C_{14}H_{10}F_2N_2O_3S$ (C, H, N, S).

*5.1.2. 1-(1-Naphtylmethyl)-thieno[3,2-c]-1,2,6-thiadiazin-4(3H)-one 2,2-dioxide (**177**)*

To a solution of thienothiadiazine dioxide **176** [48] (0.200 g, 1 mmol) in aqueous saturated solution of sodium bicarbonate (20 mL), 1-chloromethylnaphtyl chloride (0.176 g, 1 mmol) was added. The reaction mixture was refluxed for 24 h. After cooling to room temperature, the aqueous phase was washed with CH_2Cl_2 (1 × 10 mL). The aqueous phase was cooled at −4 °C and the product was isolated by filtration of aqueous phase. Purification: preparative thin layer chromatography, using CH_2Cl_2:MeOH (10:1) as eluent; yield 0.070 g (21%) as a solid; mp > 350 °C. Purity 84% (by HPLC); ^{1}H NMR (CD_3OD) δ 4.98 (s, 2H, N–CH2), 6.70 (d, 1H, $J_{H6H7} = 5.3$ Hz, H-7), 6.97–7.46 (m, 7H, Ar–H), 7.57 (d, 1H, H-6); ^{13}C NMR (CD_3OD) δ 54.5 (N–CH2), 119.7 (C-7), 120.8 (C-4a), 124.5, 125.8, 126.9, 127.0, 127.4, 127.5, 127.9, 131.5, 133.5, 135.2 (Ar–C), 134.2 (C-6), 142.6 (C-7a), 157.7 (C-4). Anal. $C_{16}H_{12}N_2O_3S_2$ (C, H, N, S).

*5.1.3. 1-[(2,5-Difluorophenyl)methyl]-benzo[4,5]thieno-[3,2-c]-1,2,6-thiadiazin-4(3H)-one 2,2-dioxide (**179**)*

To a solution of benzothienothiadiazine dioxide **178** [25] (0.254 g, 1 mmol) in aqueous saturated solution of sodium bicarbonate (20 mL), 2,5-difluorophenylmethyl bromide (0.310 g, 1.5 mmol) was added. The reaction mixture was refluxed for 3 h. After cooling to room temperature, the aqueous phase was extracted with CH_2Cl_2 (4 × 10 mL). The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure. The residue was chromatographed by preparative thin layer chromatography, using CH_2Cl_2:MeOH (8:1) as eluent; yield 0.040 g (10%) as a yellow solid; mp 224–226 °C. Purity 89% (by HPLC); ^{1}H NMR (CD_3OD) δ 4.07 (s, 2H, N–CH2), 6.39–6.52 (m, 3H, Ar–H), 7.49 (t, 1H, $J_{H7H8} = 6.9$ Hz, H-7), 7.59 (t, 1H, $J_{H8H9} = 8.1$ Hz, H-8), 7.79 (d, 1H, H-9), 7.89 (d, 1H, $J_{H6H7} = 7.3$ Hz, H-6); ^{13}C NMR (CD_3OD) δ 58.0 (N–CH2), 100.5, 109.7, 110.2, 145.7, 163.5, 163.6 (Ar–C), 123.1 (C-9), 124.1 (C-6), 125.4 (C-4a), 125.9 (C-7), 128.7 (C-8), 131.6 (C-5a), 137.1 (C-9b), 140.3 (C-9a), 158.0 (C-4). Anal. $C_{17}H_{12}F_2N_2S_2O_2$ (C, H, N, S).

*5.1.4. 1-[(3,4-Dichlorophenyl)methyl]-benzo[4,5]thieno-[3,2-c]-1,2,6-thiadiazin-4(3H)-one 2,2-dioxide (**180**)*

To a solution of benzothienothiadiazine dioxide **178** [25] (0.254 g, 1 mmol) in aqueous saturated solution of sodium bicarbonate (20 mL), 3,4-dichlorophenylmethyl chloride (0.293 g, 1.5 mmol) was added. The reaction mixture was refluxed for 3 h. After cooling to room temperature, the aqueous phase was washed with CH_2Cl_2 (4 × 10 mL). The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure. The residue was chromatographed by preparative thin layer chromatography, using CH_2Cl_2:MeOH (8:1) as eluent; yield 0.060 g (15%) as a solid; mp

227–228 °C. Purity 91% (by HPLC); ^{1}H NMR (CD_3OD) δ 4.08 (s, 2H, N–CH2), 6.91–7.03 (m, 3H, Ar–H), 7.51 (t, 1H, $J_{H7H8} = 6.9$ Hz, H-7), 7.59 (t, 1H, $J_{H8H9} = 8.1$ Hz, H-8), 7.77 (d, 1H, H-9), 7.91 (d, 1H, $J_{H6H7} = 7.3$ Hz, H-6); ^{13}C NMR (CD_3OD) δ 57.9 (N–CH2), 101.3, 128.9, 130.1, 133.02, 134.1, 141.9 (Ar–C), 122.9 (C-9), 124.3 (C-6), 125.6 (C-4a), 125.8 (C-7), 129.0 (C-8), 131.4 (C-5a), 137.1 (C-9b), 140.3 (C-9a), 158.5 (C-4). Anal. $C_{16}H_{10}Cl_2N_2S_2O_3$ (C, H, N, S).

5.1.5. (1H)-Quinazolin-2,4-dione (**181**)

To a solution of potassium isocyanate (0.081 g, 1 mmol) in 1 mL of dry toluene, methyl anthranilate (0.151 g, 1 mmol) was added. The reaction mixture was stirred at room temperature and after 3 h extracted with CH_2Cl_2 (2 × 10 mL). The organic phase was washed with an aqueous saturated solution of sodium bicarbonate, dried over sodium sulphate, and cooled at −4 °C. After 24 h, the crude solid was filtered and redissolved in 1 mL of ethanol and 1 mL of 10% NaOH. The reaction was refluxed for 1 h. Acidification of the solution with concentrate hydrochloric acid yielded 0.060 g (40%) of **181** as a white solid; mp 298–305 °C (Ref. [39], 295–300 °C). Purity 98% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.2–7.6 (m, 2H, H-6, H-8), 7.59 (m, 1H, H-7), 7.83 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 114.5 (C-4a), 115.5 (C-8), 122.5 (C-6), 127.1 (C-5), 135.1 (C-7), 141.0 (C-8a), 150.4 (C-4), 163.0 (C-2). Anal. $C_8H_6N_2O_2$ (C, H, N).

5.1.6. 3-(4-Methoxyphenyl)-(1H)-quinazolin-2,4-dione (**182**)

To a solution of 4-methoxyphenylisocyanate (0.149 g, 1 mmol) in 1 mL of dry toluene, methyl anthranilate (0.151 g, 1 mmol) was added. The reaction mixture was refluxed and after 72 h extracted with CH_2Cl_2 (2 × 10 mL). The organic phase was washed with an aqueous saturated solution of sodium bicarbonate, dried over sodium sulphate, and cooled at −4 °C. After 24 h, the crude solid was filtered and redissolved in 1 mL of ethanol and 1 mL of 10% NaOH. The reaction was refluxed for 1 h. Acidification of the solution with concentrate hydrochloric acid yielded 0.110 g (43%) of **182** as a white solid; mp 297–299 °C (Ref., 299 °C). Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 3.43 (s, 3H, OCH3), 7.10–7.26 (m, 4H, Ar–H), 7.45 (m, 1H, H-6), 7.53 (dd, 1H, $J_{H8H7} = 7.9$ Hz, $J_{H8H6} = 1.1$ Hz, H-8), 7.85 (m, 1H, H-7), 8.05 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 58.9 (OCH3), 116.3, 116.9, 123.8, 124.0, 125.2, 176.8 (Ar–C), 119.9 (C-4a), 120.7 (C-8), 128.0 (C-6), 133.1 (C-5), 140.7 (C-7), 145.3 (C-8a), 164.4 (C-4), 167.9 (C-2). Anal. $C_{15}H_{12}N_2O_3$ (C, H, N).

5.2. General procedure for the synthesis of thioxoquinazoline derivatives

To a solution of the corresponding isothiocyanate (1 mmol) in 1 mL of dry toluene, methyl anthranilate (0.151 g, 1 mmol) was added. The reaction mixture was refluxed under the indicated conditions in each case. After that, the product was isolated by filtration.

5.2.1. 3-Phenyl-2-thioxo-(1H)-quinazolin-4-one (**183**)

Reagent: phenylisothiocyanate (0.135 g, 1 mmol). Condition: 24 h. Yield 0.090 g (35%) as a white solid; mp 300–302 °C (Ref. [49], 301 °C). Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.20–7.60 (m, 7H, Ar–H, H-6, H-8), 7.80 (m, 1H, H-7), 8.06 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 115.8 (C-4a), 116.3 (C-8), 124.5 (C-6), 127.5 (C-5), 128.2, 129.0, 129.1, 139.4 (Ar–C), 135.7 (C-7), 139.9 (C-8a), 159.9 (C-4), 176.2 (C-2). Anal. $C_{14}H_{10}N_2SO$ (C, H, N, S).

5.2.2. 3-(4-Methoxyphenyl)-2-thioxo-(1H)-quinazolin-4-one (**184**)

Reagent: 4-methoxyphenylisothiocyanate (0.165 g, 1 mmol). Condition: 72 h. Yield 0.070 g (24%) as a white solid; mp 274–276 °C (Ref. [43], 275 °C). Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 3.76 (s, 3H, OCH_3), 6.83–7.59 (m, 6H, Ar–H, H-6, H-8), 7.77 (m, 1H, H-7), 7.93 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.4$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 55.7 (OCH_3), 115.7 (C-4a), 115.2 (C-8), 116.5, 116.7, 130.0, 130.8, 139.3, 159.3 (Ar–C), 125.5 (C-6), 127.6 (C-5), 135.8 (C-7), 140.2 (C-8a), 159.7 (C-4), 175.8 (C-2). Anal. $C_{15}H_{12}N_2SO_2$ (C, H, N, S).

5.2.3. 3-(2,6-Difluorophenyl)-2-thioxo-(1H)-quinazolin-4-one (**185**)

Reagent: 2,6-difluorophenylisothiocyanate (0.171 g, 1 mmol). Condition: 4 days. Yield 0.160 g (55%) as a white solid; mp 262–263 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.30–7.66 (m, 5H, Ar–H, H-6, H-8), 7.85 (m, 1H, H-7), 8.01 (dd, 1H, $J_{H5H6} = 7.7$ Hz, $J_{H5H7} = 0.9$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 112.5, 112.7, 115.2, 131.8, 156.5, 159.0 (Ar–C), 115.5 (C-4a), 116.5 (C-8), 125.5 (C-6), 127.9 (C-5), 137.0 (C-7), 140.0 (C-8a), 159.8 (C-4), 175.0 (C-2). Anal. $C_{14}H_8F_2N_2SO$ (C, H, N, S).

5.2.4. 3-(2,3,4-Trifluorophenyl)-2-thioxo-(1H)-quinazolin-4-one (**186**)

Reagent: 2,3,4-trifluorophenylisothiocyanate (0.189 g, 1 mmol). Condition: 14 days. Yield 0.200 g (67%) as a white solid; mp 260–261 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.37–7.56 (m, 4H, Ar–H, H-6, H-8), 7.83 (m, 1H, H-7), 7.99 (dd, 1H, $J_{H5H6} = 7.2$ Hz, $J_{H5H7} = 0.9$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 113.0, 124.4, 126.5, 145.3, 148.9, 152.2 (Ar–C), 115.8 (C-4a), 116.3 (C-8), 125.2 (C-6), 127.8 (C-5), 136.5 (C-7), 139.9 (C-8a), 159.6 (C-4), 175.5 (C-2). Anal. $C_{14}H_7F_3N_2SO$ (C, H, N, S).

5.2.5. 3-(2-Bromophenyl)-2-thioxo-(1H)-quinazolin-4-one (**187**)

Reagents: 2-bromophenylisothiocyanate (0.214 g, 1 mmol). Conditions: 36 h. Yield 0.170 g (52%) as a white solid; mp 252–254 °C (Ref. [44], 256 °C). Purity 98% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.20–7.80 (m, 7H, Ar–H, H-6, H-7, H-8),

7.79 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 115.8 (C-4a), 116.0 (C-8), 122.5, 128.8, 130.4, 131.4, 132.9, 138.3 (Ar–C), 124.7 (C-6), 127.6 (C-5), 136.9 (C-7), 139.8 (C-8a), 159.1 (C-4), 175.2 (C-2). Anal. $C_{14}H_9BrN_2SO$ (C, H, N, S).

*5.2.6. 3-Benzyl-2-thioxo-(1H)-quinazolin-4-one (**188**)*

Reagent: benzylisothiocyanate (0.149 g, 1 mmol). Condition: 24 h. Yield 0.130 g (50%) as a yellow solid; mp 231–233 °C (Ref. [45], 231–233 °C). Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 5.22 (s, 2H, CH2), 7.18–7.26 (m, 7H, Ar–H, H-6, H-8), 7.74 (m, 1H, H-7), 7.81 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 50.4 (CH2), 116.3 (C-8), 116.6 (C-4a), 124.5 (C-6), 126.7, 127.7, 129.3, 140.8 (Ar–C), 127.9 (C-5), 135.8 (C-7), 140.6 (C-8a), 159.7 (C-4), 175.2 (C-2). Anal. $C_{15}H_{12}N_2SO$ (C, H, N, S).

*5.2.7. 3-(1-Naphtyl)-2-thioxo-(1H)-quinazolin-4-one (**189**)*

Reagent: 1-naphtylisothiocyanate (0.185 g, 1 mmol). Condition: 7 days. Yield 0.050 g (18%) as a white solid; mp 226–228 °C (Ref. [42], 228 °C). Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.15–7.79 (m, 9H, Ar–H, H-6, H-8), 7.74 (m, 1H, H-7), 7.90 (dd, 1H, $J_{H5H6} = 7.9$ Hz, $J_{H5H7} = 1.1$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 115.7 (C-4a), 115.9 (C-8), 124.1, 124.5, 124.9, 125.7, 125.8, 126.5, 126.6, 128.2, 134.6, 135.4, (Ar–C), 124.5 (C-6), 127.8 (C-5), 135.7 (C-7), 139.6 (C-8a), 159.7 (C-4), 175.7 (C-2). Anal. $C_{18}H_{12}N_2SO$ (C, H, N, S).

*5.2.8. 3-Methyl-2-thioxo-(1H)-quinazolin-4-one (**190**)*

Reagent: methylisothiocyanate (0.073 g, 1 mmol). Condition: 72 h. Yield 0.090 g (46%) as a yellow solid; mp 262–264 °C (Ref. [50], 259–260 °C). Purity 90% (by HPLC); ^{1}H NMR (CD_3OD) δ 3.45 (s, 3H, CH_3), 7.30–7.39 (m, 2H, H-6, H-8), 7.73 (m, 1H, H-7), 7.95 (dd, 1H, $J_{H5H6} = 8.0$ Hz, $J_{H5H7} = 0.7$ Hz, H-5); ^{13}C NMR (CD_3OD) δ 33.6 (CH_3), 115.6 (C-4a), 115.9 (C-8), 124.7 (C-6), 127.6 (C-5), 135.6 (C-7), 139.6 (C-8a), 159.9 (C-4), 175.7 (C-2). Anal. $C_9H_8N_2SO$ (C, H, N, S).

5.3. General procedure for the synthesis of thienopyrimidinone derivatives

To a solution of the corresponding isothiocyanate (1 mmol) in 1 mL of dry toluene, 3-amino-thiophene-2-carboxylic acid methyl ester [48] (0.157 g, 1 mmol) was added. The reaction mixture was refluxed under the indicated conditions in each case. After that, the product was isolated by filtration.

*5.3.1. 3-Methyl-2-thioxo-(1H)-thieno[3,2-d]pyrimidin-4-one (**191**)*

Reagent: methylisothiocyanate (0.073 g, 1 mmol). Condition: 5 days. Yield 0.040 g (21%) as a brown solid; mp 234–236 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 2.97 (s, 3H, CH_3), 7.01 (d, 1H, $J_{H6H7} = 5.1$ Hz, H-7), 8.15 (d, 1H, H-6); ^{13}C NMR (CD_3OD) δ 33.6 (CH_3), 145.3 (C-4a), 115.0 (C-7), 117.5 (C-6), 137.5 (C-7a), 156.8 (C-4), 175.6 (C-2). Anal. $C_7H_6N_2SO_2$ (C, H, N, S).

*5.3.2. 3-Phenyl-2-thioxo-(1H)-thieno[3,2-d]pyrimidin-4-one (**192**)*

Reagent: phenylisothiocyanate (0.135 g, 1 mmol). Condition: 5 days. Yield 0.030 g (13%) as a white solid; mp 265–267 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.07 (d, 1H, $J_{H6H7} = 5.1$ Hz, H-7), 7.23–7.78 (m, 5H, Ar–H), 7.84 (d, 1H, H-6); ^{13}C NMR (CD_3OD) δ 115.7 (C-7), 117.7 (C-6), 126.0, 128.6, 129.3, 139.5 (Ar–C), 137.9 (C-7a), 145.6 (C-4a), 157.0 (C-4), 176.7 (C-2). Anal. $C_{12}H_8N_2SO_2$ (C, H, N, S).

*5.3.3. 3-(2,6-Difluorophenyl)-2-thioxo-(1H)-thieno[3,2-d]pyrimidin-4-one (**193**)*

Reagent: 2,6-difluorophenylisothiocyanate (0.171 g, 1 mmol). Condition: 17 days. Yield 0.005 g (2%) as a white solid; mp 270–272 °C. Purity 98% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.11 (d, 1H, $J_{H6H7} = 5.1$ Hz, H-7), 7.21–8.30 (m, 3H, Ar–H), 7.84 (d, 1H, H-6); ^{13}C NMR (CD_3OD) δ 112.5, 112.7, 126.0, 159.9 (Ar–C), 114.4 (C-7), 117.9 (C-6), 139.5 (C-7a), 146.6 (C-4a), 156.6 (C-4), 175.5 (C-2). Anal. $C_{12}H_6F_2N_2SO_2$ (C, H, N, S).

5.4. General procedure for the synthesis of benzothienopyrimidinone derivatives

To a solution of the corresponding isothiocyanate (1 mmol) in 1 mL of dry toluene, 3-amino-benzo[*b*]thiophene-2-carboxylic acid methyl ester [51] (0.207 g, 1 mmol) was added. The reaction mixture was refluxed under the indicated conditions in each case. After that, the product was isolated by filtration.

*5.4.1. 3-Phenyl-2-thioxo-(1H)-benzo[4,5]thieno[3,2-d]-pyrimidin-4-one (**194**)*

Reagent: phenylisothiocyanate (0.135 g, 1 mmol). Condition: 5 days. Yield 0.040 g (14%) as a yellow solid; mp 255–256 °C. Purity 95% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.24–7.47 (m, 5H, Ar–H), 7.56 (m, 1H, H-8), 7.64 (t, 1H, $J_{H7H8} = 6.0$ Hz, H-7), 8.10 (d, 1H, $J_{H6H7} = 6.3$ Hz, H-6), 8.60 (d, 1H, $J_{H8H9} = 5.4$ Hz, H-9); ^{13}C NMR (CD_3OD) δ 114.5 (C-4a), 124.0 (C-9), 124.0 (C-6), 125.6 (C-8), 128.0, 129.0, 139.7 (Ar–C), 129.3 (C-7), 129.9 (C-9a), 140.5 (C-5a), 142.6 (C-9b), 157.7 (C-4), 177.0 (C-2). Anal. $C_{16}H_{10}N_2S_2O$ (C, H, N, S).

*5.4.2. 3-(2,6-Difluorophenyl-2-thioxo-(1H)-benzo[4,5]-thieno[3,2-d]-pyrimidin-4-one (**195**)*

Reagent: 2,6-difluorophenylisothiocyanate (0.171 g, 1 mmol). Condition: 24 h. Yield 0.040 g (12%) as a yellow solid; mp 280–282 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.22–7.33 (m, 3H, Ar–H), 7.52–7.62 (m, 2H, H-7, H-8), 8.14 (d, 1H, $J_{H6H7} = 8.06$ Hz, H-6), 8.60 (d, 1H, H-9); ^{13}C NMR (CD_3OD) δ 117.6, 130.8, 142.6, 160.3 (Ar–C), 123.1 (C-4a), 124.0 (C-6), 124.2 (C-9), 125.7 (C-8), 128.6 (C-9a), 129.7 (C-7), 140.9 (C-5a), 143.7 (C-9b), 156.4 (C-4), 175.9 (C-2). Anal. $C_{16}H_8F_2N_2S_2O$ (C, H, N, S).

*5.4.3. 3-(2,3,4-Trifluorophenyl-2-thioxo-(1H)-benzo[4,5]thieno[3,2-d]-pyrimidin-4-one (**196**)*

Reagent: 2,3,4-trifluorophenylisothiocyanate (0.189 g, 1 mmol). Condition: 17 days. Yield 0.030 g (9%) as a yellow solid; mp 268–269 °C. Purity 99% (by HPLC); ^{1}H NMR (CD_3OD) δ 7.15–7.38 (m, 2H, Ar–H), 7.49–7.65 (m, 2H, H-7, H-8), 8.14 (d, 1H, $J_{H6H7} = 8.02$ Hz, H-6), 8.60 (d, 1H, H-9); ^{13}C NMR (CD_3OD) δ 115.3, 118.8, 130.1, 130.2, 131.4, 167.0 (Ar–C), 123.1 (C-4a), 124.2 (C-9), 124.3 (C-6), 125.5 (C-8), 128.7 (C-9a), 129.6 (C-7), 140.8 (C-5a), 143.8 (C-9b), 156.4 (C-4), 175.8 (C-2). Anal. $C_{16}H_7F_3N_2S_2O$ (C, H, N, S).

5.5. Measurement of PDE activities

PDE3A (purified from human platelets), PDE4B (human recombinant) and PDE7A (human recombinant) activities were monitored by measuring the hydrolysis of [^{3}H]-cAMP to [^{3}H]-AMP using a PDE–SPA kit (Amersham). Extracts containing the corresponding human phosphodiesterases were incubated in ''low binding'' plates (Costar 3604) for 60 min at room temperature. The assay mixture (80 μL) contains 15 nM [^{3}H]-cAMP (1 μCi/mL) in the assay buffer (50 mM tris, pH 7.5, 8.3 mM $MgCl_2$, 1.7 mM EGTA) and 10 μL of test compound. These compounds were resuspended in DMSO (final DMSO concentration 5% (v/v)) and tested at different concentrations varying from 1 mM to 1 nM to calculate an IC_{50} and/or % activity inhibition. These dilutions were done in 96-well plates.

Hydrolysis of [^{3}H]-cAMP was initiated by adding 10 μL of a solution containing the corresponding phosphodiesterase extract, and the plate was then incubated under agitation at room temperature. The reaction was stopped after 60 min (with 10–20% substrate conversion) by addition of 50 μL phosphodiesterase scintillation proximity assay (SPA) beads. All reactions were carried out in duplicate. After incubation the reaction was stopped with 50 μL (0.89 mg) of PDE SPA beads (Amersham Pharmacia Biotech), and the resulting mixture was allowed to settle for 20 min before counting in a microtitre plate counter.

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Appendix. Supplementary data

Theoretical basis of CODES and structures of the database. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2007.10.027.

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