Synthesis and In Vitro Antiplatelet Activity of New 4-(1-Piperazinyl)coumarin Derivatives. Human Platelet Phosphodiesterase 3 Inhibitory Properties of the Two Most Effective Compounds Described and Molecular Modeling Study on Their Interactions with Phosphodiesterase 3A Catalytic Site

Giorgio Roma,*,† Mario Di Braccio,† Giancarlo Grossi,† Daniela Piras,† Giuliana Leoncini,‡ Debora Bruzzese,‡ Maria Grazia Signorello,‡ Paola Fossa,† and Luisa Mosti†

Dipartimento di Scienze Farmaceutiche, Università di Genova, Viale Benedetto XV n. 3, I-16132 Genoa, Italy, and Dipartimento di Medicina Sperimentale, Sezione Biochimica, Università di Genova, Viale Benedetto XV n. 1, I-16132 Genoa, Italy

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The synthesis and in vitro antiplatelet activity significant data of coumarin derivatives **5i**−**x** and quinolin-2(1*H*)-one derivatives **22a**,**b**, as well as the corresponding structure−activity relationships are described. The recently reported 8-methyl-4-(1-piperazinyl)-7-(3-pyridylmethoxy)coumarin **5f** and its potent 7-(2-morpholinoethoxy)-substituted new analogue **5u** were notably more effective inhibitors of pure human platelet PDE3 than milrinone and cilostazol: these data were related, through a molecular modeling study, with the molecular interactions of the four compounds with the human PDE3A catalytic site.

Introduction

Platelets play a very important role in haemostatic and thrombotic processes. Platelet function may be modulated by many different agents, of which the second messenger cAMP is a strong inhibitor of platelet activation. The intracellular cAMP levels are controlled through its synthesis rate by adenylate cyclase and/or its hydrolysis by phosphodiesterases (PDEs[a]).[1,2] Platelets contain three classes of PDEs: PDE2, PDE3, and PDE5. The PDE3 isoform is the most abundant, exhibits low K_m for cAMP, is competitively inhibited by cGMP, and is localized in the cytosolic fraction. The above three PDE forms are differently affected by various PDE inhibitors. Some of these inhibitors are selective for one PDE isoform. In particular, cilostamide, cilostazol, enoximone, imazodan, and milrinone[3] are specific inhibitors of the PDE3 isoform. As this isoform accounts for 80−90% of the total platelet PDE, the inhibition of this enzyme produces a strong antiplatelet effect. As a result, a better structural knowledge of the PDE3 catalytic and allosteric sites should permit the development of more potent and selective drugs. Thus, the synthesis of new specific inhibitors of platelet PDE3 appears to be an effective route to novel platelet antiaggregating agents.

In the course of our studies aimed at obtaining new human platelet aggregation inhibitors, we synthesized and tested, for their in vitro activity, numerous substituted 2-aminochromones **1**,[4−6] their benzo-fused derivatives **2**−**4**,[6,7] and the corresponding 4-aminocoumarin derivatives **5**−**8**,[6−8] as well as the 1,2-fused pyrimidine amino derivatives **9**−**11**[7,9−11] and **12**−**14**,[10] isosteric analogues of compounds **1**−**3** and **5**−**7**, respectively (Figure 1). In fact, the platelet antiaggregating properties of compounds **1**, the first ones examined, were proved to arise from their inhibitory activity on PDE3.[12] Compounds **15**, whose structures were derived from that of the very active compound **9a**[10] (Table 1) by a suitable modification of its pyridine ring, were also synthesized and tested for their antiplatelet activity[7,11] (Figure 1).

On the whole, the antiplatelet activity data shown in vitro by the above-mentioned compounds **1**−**16** toward all the platelet aggregation inducers used [i.e., adenosine diphosphate (ADP), collagen, and the Ca^{2+} ionophore A23187 (calcimycin)], suggest the following remarks: (1) The structures of compounds **1**−**15** are characterized by the presence of a β-enaminone moiety whose importance for the activity, in this structural field, was clearly confirmed, for instance, by the impressive activity difference between compounds **9a**[10] and **16**[11] (Figure 1, Table 1). (2) In compounds **1**−**11** and **15**, the 1-piperazinyl group proved to be the most effective amino substituent of all those used, but the 1-piperazinyl derivatives of compounds **12**−**14** could not be obtained. (3) However, the antiplatelet activity potency of the β-enaminonic 1-piperazinyl derivatives **1**−**11** and **15** is significantly affected by the structure of the supporting cyclic system: see, for instance (Table 1), the data of 2-(1-piperazinyl)chromone **1a**, its isomer **5a**, and its potent isosteric analogue 2-(1-piperazinyl)-4*H*-pyrido[1,2-*a*]pyrimidin-4-one **9a**. (4) On the other hand, the coumarin derivative **5a** was the most suitable compound for increasing its activity potency by the introduction of further proper substituents (e.g., compounds **5a**−**f**, Table 1). In fact, the 8-methyl-4-(1-piperazinyl)-7-(3-pyridylmethoxy)coumarin **5f** is the most potent in vitro antiplatelet agent of all the compounds **1**−**15** previously synthesized by us.[8] (5) In regard to the mechanism involved in the antiplatelet activity of these compounds, the 2-(diethylamino)-7-hydroxychromone [**1**: $NRR^1 = N(C_2H_5)_2$, R^2 = 7-OH, R^3 = H],[12] the 1,2-fused pyrimidine derivative **9a**[13] as well as the coumarin derivative **5f**[14] were proved to increase platelet cAMP levels through the specific inhibition of PDE3, in accordance with their common structural features. In the case of **5f** antiplatelet activity, its proven ability to increase nitric oxide formation must also be pointed out.[14]

Starting from these results, we have pursued our studies in this field to obtain further significantly active antiplatelet agents and to improve our knowledge of the structure−activity relationships (SAR). Thus, in the present paper we report the

* To whom correspondence should be addressed. Phone: +390103538374. Fax: +390103538358. E-mail: roma@unige.it.
† Dipartimento di Scienze Farmaceutiche.
‡ Dipartimento di Medicina Sperimentale.
[a] Abbreviations: PDE, phosphodiesterase; ASA, acetylsalicylic acid; PRP, platelet-rich plasma.

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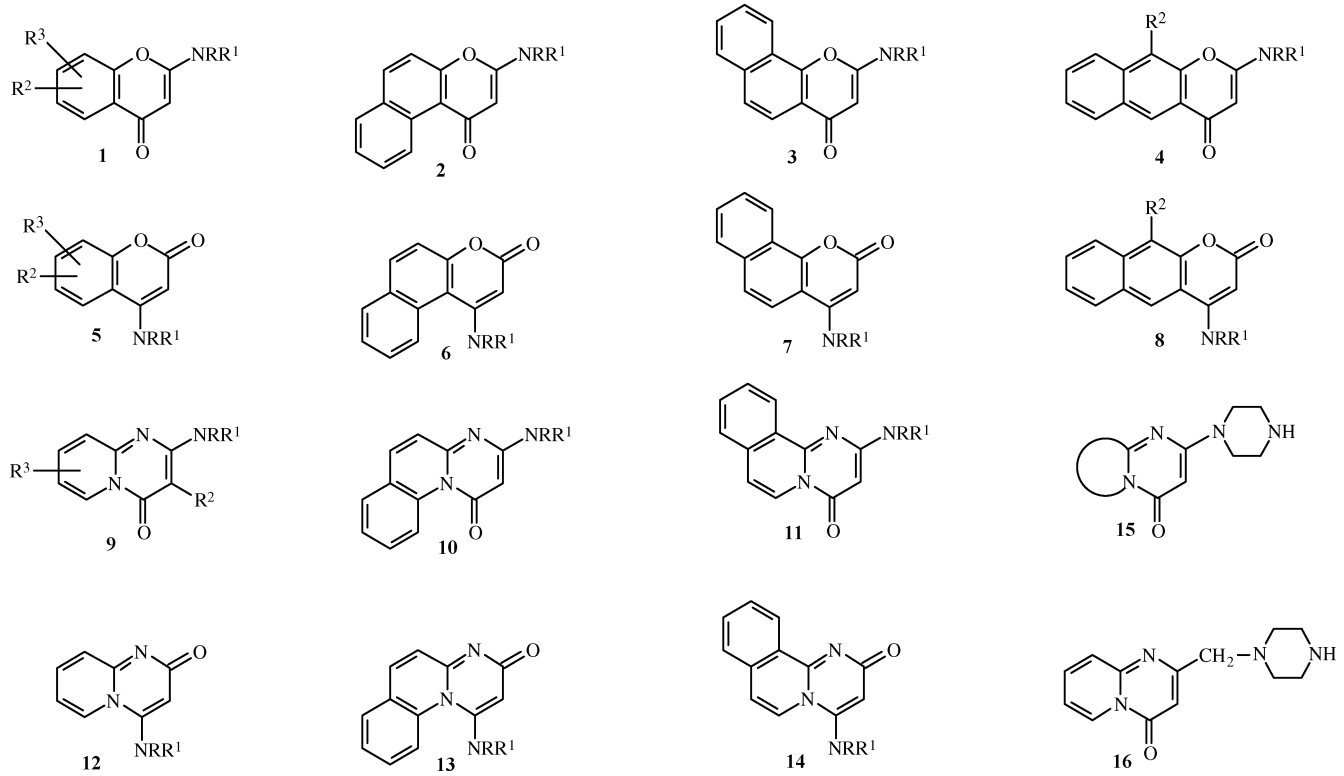


Figure 1. Structures of 2-aminochromone and 4-aminocoumarin bicyclic and tricyclic derivatives **1**−**4** and **5**−**8**, pyrido[1,2-*a*]pyrimidine bicyclic and tricyclic derivatives **9**−**11**, **16**, and **12**−**14**, and 1,2-fused pyrimidine derivatives **15**.

synthesis and biological evaluation of several properly substituted 4-(1-piperazinyl)coumarins (**5i**−**w**), their analogue **5x**, and two methyl-substituted 4-(1-piperazinyl)quinolin-2(1*H*)-ones (**22a**,**b**), their isosteric analogues. Most of these compounds have been designed through the molecular modification of **5f**, taken as a lead.[8] Furthermore, a molecular modeling study is described concerning both compound **5f** and the novel very active one **5u**, aimed at relating the human PDE3 inhibitory activity data of these compounds with their profiles of interaction with the enzyme.

Chemistry

The synthetic routes to the 4-(1-piperazinyl)coumarin derivatives **5i**−**w**, their analogue **5x**, and 4-(1-piperazinyl)quinolin-2(1*H*)-one derivatives **22a**,**b** are shown in Scheme 1.

The dihydroxyacetophenones **17a**−**c** were treated with the proper alkyl halides to give the corresponding alkoxy derivatives **18a**−**g**,**i**−**n** (KOH/anhydrous K_2CO_3, dry 2-butanone at reflux; yields 32−85%) and **18h**,**o** (anhydrous K_2CO_3, 160 °C; yields 53% and 30%, respectively), whose cyclocondensation with diethyl carbonate in the presence of Na (Dowtherm A, 160 °C) afforded the 4-hydroxycoumarin derivatives **19a**−**o** (yields 50−91%). By treating compounds **19a**−**m**,**o** with a large excess of piperazine at 160 °C, the corresponding substituted 4-(1-piperazinyl)coumarins **5i**−**u**,**w** (yields 30−69%) were then obtained. Compound **5v** was more conveniently obtained (85%) by treating with excess piperazine (ethanol at reflux) the 4-chlorocoumarin derivative **20a**, previously prepared (53%) by heating (120 °C) the properly substituted 4-hydroxycoumarin **19n** with excess phosphorus oxychloride in the presence of triethylamine. Compound **5x** was afforded (68%) by the reaction of the corresponding 4-chlorocoumarin **20b**[8] with excess 4-aminopiperidine (ethanol at room temperature).

The 4-(1-piperazinyl)quinolin-2(1*H*)-one derivatives **22a** (90%) and **22b** (78%) were prepared by treating with an excess of piperazine the corresponding 4-chloro derivatives **21a**[15] (ethanol at reflux) and **21b**[16] (ethylene glycol, 150 °C), respectively.

The results of elemental analyses, and IR and ^{1}H NMR spectral data are consistent with the structures assigned to the compounds described in the present paper, and the spectral data agree with the ones previously reported by us for analogous compounds.[6−8]

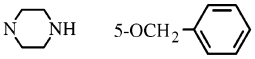
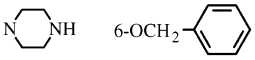
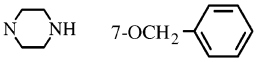
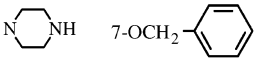
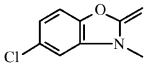
Biological Results and Discussion

The novel compounds **5i**−**x** and **22a**,**b** described in the present paper, as well as the reference compounds acetylsalicylic acid (ASA), cilostazol, and milrinone, were tested in vitro for their inhibitory activity on the human platelet aggregation induced in platelet-rich plasma (PRP) by ADP, collagen, and the Ca^{2+} ionophore A23187 (see Experimental Section). The IC_{50} values obtained are reported in Table 2.

Taking into account the SAR considerations reported in the Introduction and the activity improvement afforded by the 8-methyl substituent (see, for instance, the IC_{50} values of compounds **5d** and **5e**, Table 1), the molecular modification of the lead compound **5f** has now mainly concerned its 7-alkoxy substituent. Furthermore, the activity increase afforded by the isosteric replacement of the 7-benzyloxy group (**5e**) with the 7-(3-pyridylmethoxy) one (**5f**) must be pointed out (see Table 1). Thus, we have now turned our attention to the synthesis and biological evaluation of novel 4-(1-piperazinyl)-8-methylcoumarins **5** with 7-alkoxy substituents containing proper heteroatoms whose unshared electron pairs are available to interact with the PDE3 active site (see Molecular Modeling Studies).

Considering the biological results reported in Table 2, the following remarks can be made: (1) The comparison of the IC_{50} values of compounds **5g** and **5h**[8] (Table 1) with those of the new compounds **5l**, **5m**, and **5n**, **5o**, respectively (Table 2),

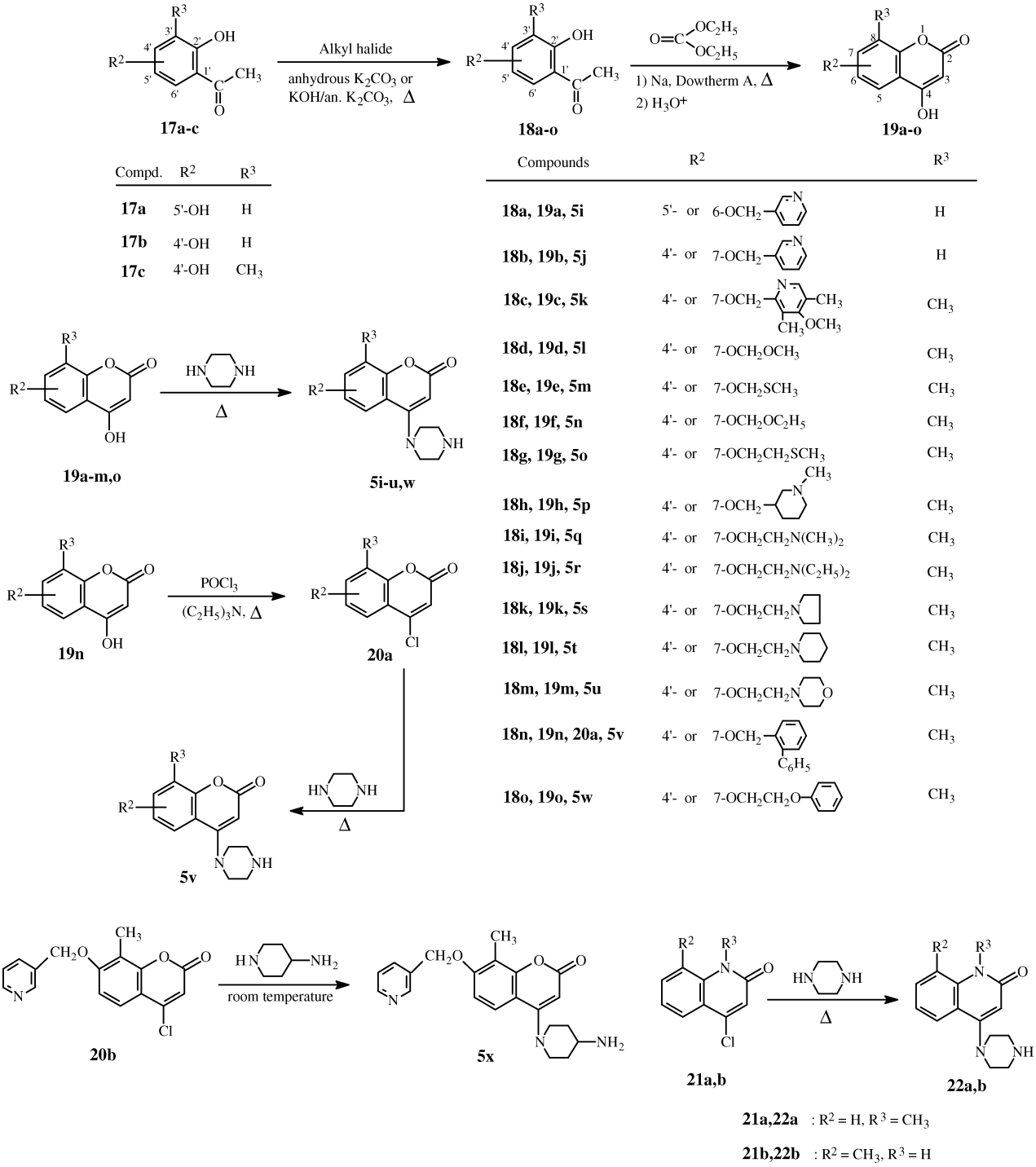
Table 1. In Vitro Inhibitory Activity of Some Significant Previously Described Compounds[a] (Figure 1) on Human Platelet Aggregation Induced in PRP[b] by ADP, Collagen, and A23187[c]

Compound	NRR[1]	R[2]	R[3]		IC_{50} (μM) ± SD		
					ADP (5.0 μM)	Collagen (5.0 μg/mL)[d]	A23187 (20.0 μM)
1a		H	H	-	56 ± 13	60 ± 12	240 ± 90
5a		H	H	-	49 ± 14	36 ± 10	250 ± 38
5b		5-OCH_2	H	-	187 ± 42	144 ± 40	429 ± 105
5c		6-OCH_2	H	-	23.6 ± 4.7	34.2 ± 3.3	116 ± 12.3
5d		7-OCH_2	H	-	16.3 ± 4.1	24.2 ± 5.1	78.8 ± 10.3
5e		7-OCH_2	8-CH_3	-	1.9 ± 0.2	1.8 ± 0.4	1.1 ± 0.2
5f		7-OCH_2	8-CH_3	-	0.98 ± 0.36	0.51 ± 0.12	1.42 ± 0.43
5g		7-$O(CH_2)_2CH_3$	8-CH_3	-	2.95 ± 0.78	2.58 ± 0.46	7.77 ± 1.36
5h		7-$O(CH_2)_3CH_3$	8-CH_3	-	2.79 ± 1.30	1.92 ± 0.96	5.03 ± 1.02
9a		H	H	-	6 ± 1.8	3.6 ± 1.2	19 ± 9
15a	-	-	-		3.6 ± 1.2	8.8 ± 5.6	13.0 ± 5
16	-	-	-	-	>1000	>1000	>1000

[a] Compounds **1a**, **5a**, ref 6; compounds **5b**−**d**,**f**−**h**, ref 8; compounds **5e**, **15a**, ref 7; compound **9a**, ref 10; and compound **16**, ref 11. [b] PRP = platelet-rich plasma. [c] See Introduction. [d] 10.0 μg/mL for compounds **1a**, **5a**.

clearly indicates that the isosteric replacement of a CH_2 group in the 7-propoxy (**5g**) and 7-butoxy (**5h**) substituents with proper heteroatoms (O, S) significantly increases the activity of the corresponding compounds. The notably lower activity of compound **5w** could be due to the delocalization of the oxygen unshared electron pair into the phenyl ring. (2) On the other hand, the replacement in the **5g** 7-propoxy substituent of the CH_3 group with a disubstituted NH_2 group affords variably less active compounds (**5q**−**s**), in spite of the presence of the nitrogen unshared electron pair. This behavior can be justified by the steric hindrance of the N-substituents. In fact, the 7-(2-morpholinoethoxy)-substituted compound **5u**, whose morpholine oxygen atom is sterically slightly hindered, showed an overall very potent in vitro antiplatelet activity, significantly more potent than that of its isosteric analogue **5t** (see Table 2). Also, in the case of compounds **5k** and **5p**, the pyridine and piperidine rings, respectively, present in their 7-alkoxy groups, show further substituents so that the relatively good activities of these compounds are likely conditioned by steric hindrance. (3) The remarkable difference between the IC_{50} values obtained for compounds **5x** (Table 2) and **5f** (Table 1) further confirms the importance of both the β-enaminone moiety and 1-piperazinyl (as its amino group) for the antiplatelet activity potency in this structural field. (4) As we previously observed in the case of the 5-, 6-, and 7-benzyloxy-substituted 4-(1-piperazinyl)coumarins **5b**−**d** (Table 1), also the IC_{50} values of the 6- and 7-(3-pyridylmethoxy)-substituted 4-(1-piperazinyl)coumarins **5i** and **5j** (Table 2), clearly indicate that the 7-alkoxy substituent is the most effective for the antiplatelet activity of compounds **5**. Moreover, the notable difference between the IC_{50} values of the 8-methyl-substituted compound **5f** (Table 1) and those of compound **5j** (Table 2) significantly confirms the importance

Scheme 1. Synthetic Routes to the Coumarin Derivatives **5i**−**x** and to the Quinolin-2(1*H*)-one Derivatives **22a**,**b**



Compd.	R^2	R^3
17a	5'-OH	H
17b	4'-OH	H
17c	4'-OH	CH_3

Compounds	R^2	R^3
18a, 19a, 5i	5'- or 6-OCH_2-(N-pyridyl)	H
18b, 19b, 5j	4'- or 7-OCH_2-(N-pyridyl)	H
18c, 19c, 5k	4'- or 7-OCH_2-(N-pyridyl, 2-CH_3, 3-OCH_3)	CH_3
18d, 19d, 5l	4'- or 7-OCH_2OCH_3	CH_3
18e, 19e, 5m	4'- or 7-OCH_2SCH_3	CH_3
18f, 19f, 5n	4'- or 7-$OCH_2OC_2H_5$	CH_3
18g, 19g, 5o	4'- or 7-$OCH_2CH_2SCH_3$	CH_3
18h, 19h, 5p	4'- or 7-OCH_2-(N-CH_3-piperidyl)	CH_3
18i, 19i, 5q	4'- or 7-$OCH_2CH_2N(CH_3)_2$	CH_3
18j, 19j, 5r	4'- or 7-$OCH_2CH_2N(C_2H_5)_2$	CH_3
18k, 19k, 5s	4'- or 7-OCH_2CH_2-(N-pyrrolidyl)	CH_3
18l, 19l, 5t	4'- or 7-OCH_2CH_2-(N-piperidyl)	CH_3
18m, 19m, 5u	4'- or 7-OCH_2CH_2-(N-morpholyl)	CH_3
18n, 19n, 20a, 5v	4'- or 7-OCH_2-(C_6H_5)	CH_3
18o, 19o, 5w	4'- or 7-OCH_2CH_2O-(C_6H_5)	CH_3

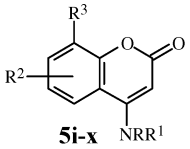
of the 8-methyl substituent for the platelet antiaggregating activity of compounds **5**. (5) With regard to the first quinoline derivatives **22** tested in these studies, as analogues of compounds **5**, the antiplatelet activity data of both the 8-methyl-4-(1-piperazinyl)quinolin-2(1*H*)-one **22b** and its 1-methyl-substituted isomer **22a** (Table 2) are on the whole less interesting than those of 4-(1-piperazinyl)coumarin **5a** (Table 1), similarly devoid of the 7-alkoxy substituent.

In conclusion, the novel compounds **5k**−**o**,**t**,**u** proved to be very interesting in vitro inhibitors of human platelet aggregation toward all the platelet aggregation inducers used and, along with **5p**,**q**, notably more active than cilostazol and milrinone. The most potent ones (i.e., **5m**, **5o**, and **5u**) afforded some IC_{50} values in the nanomolar range (Table 2).

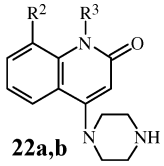
To closely investigate the mechanism of action of the compounds described and discussed in the present report, we have selected **5f**[8] and **5u**, the most potent antiplatelet agents until now synthesized by us, and evaluated their inhibitory activity on the PDE3 isolated from human platelets.

The data in Table 3 demonstrate that **5f** is a more potent PDE3 inhibitor than **5u**. In addition, **5f** and **5u** are both much more effective than the two reference agents milrinone and cilostazol, proving to be potent inhibitors of human platelet PDE3 and thus able to exert an in vitro antiplatelet activity clearly more powerful than that of these reference agents (see Tables 1, 2). However, further in vivo experiments are necessary to understand if they might exert beneficial effects in patients with cardiovascular diseases.

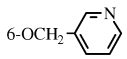
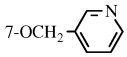
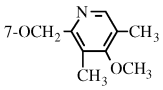
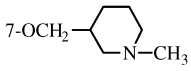
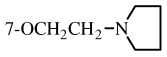
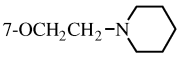
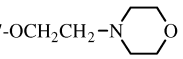
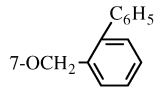
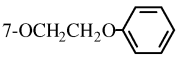
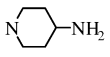
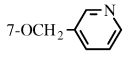
Table 2. In Vitro Inhibitory Activity of the New Compounds **5i**−**x** and **22a**,**b** on Platelet Aggregation Induced in Human PRP by ADP, Collagen, and A23187



5i-x



22a,b

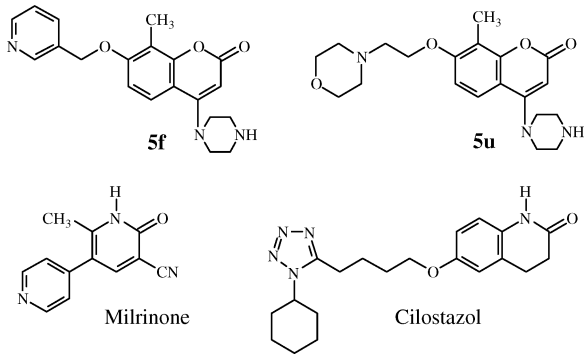
Compound	NRR^1	R^2	R^3	IC_{50} (μM) ± SD [a] ADP (5.0 μM)	Collagen (5.0 μg/mL)	A23187 (20.0 μM)
5i			H	38.0 ± 10.0	18.3 ± 5.1	36.0 ± 11.0
5j			H	21.3 ± 5.5	6.6 ± 1.8	18.9 ± 1.3
5k			CH_3	3.3 ± 1.1	2.1 ± 0.7	5.8 ± 1.2
5l		7-OCH_2OCH_3	CH_3	2.3 ± 0.21	1.3 ± 0.5	3.5 ± 1.5
5m		7-OCH_2SCH_3	CH_3	0.9 ± 0.36	0.44 ± 0.041	3.4 ± 0.28
5n		7-$OCH_2OC_2H_5$	CH_3	1.8 ± 0.3	1.1 ± 0.2	1.9 ± 0.4
5o		7-$OCH_2CH_2SCH_3$	CH_3	1.5 ± 0.2	0.8 ± 0.3	1.5 ± 0.2
5p			CH_3	4.7 ± 1.8	6.4 ± 1.4	16.4 ± 3.8
5q		7-OCH_2CH_2-$N(CH_3)_2$	CH_3	3.4 ± 0.78	3.8 ± 1.2	13.7 ± 2.2
5r		7-OCH_2CH_2-$N(C_2H_5)_2$	CH_3	11.8 ± 1.2	7.1 ± 2.2	10.7 ± 3.4
5s			CH_3	11.8 ± 3.2	10.4 ± 1.4	13.3 ± 2.8
5t			CH_3	6.1 ± 2.1	1.9 ± 0.1	5.7 ± 2.3
5u			CH_3	1.4 ± 0.50	0.284 ± 0.01	2.5 ± 0.9
5v			CH_3	17.3 ± 2.5	7.7 ± 2.2	23.0 ± 7.1
5w			CH_3	9.4 ± 2.3	4.2 ± 0.6	14.3 ± 2.7
5x			CH_3	156 ± 22	169 ± 24	175 ± 32
22a	-	H	CH_3	74.8 ± 10.0	169 ± 69	218.8 ± 15.0
22b	-	CH_3	H	95 ± 15	72 ± 10	163 ± 27
ASA				>1000	183 ± 4	>1000
Cilostazol				73 ± 19	40 ± 9	109 ± 35
Milrinone				11 ± 4	12 ± 4	14 ± 5

[a] IC_{50} = compound concentration that inhibits platelet aggregation by 50%.

Molecular Modeling Studies

To further rationalize the biological results, a molecular docking study was carried out on the very effective PDE3 inhibitors **5f**[8] and **5u**, plus milrinone and cilostazol, which were used as reference compounds in the biological assays. In this way we evaluated by an in silico approach the different molecular interactions displayed by these compounds and thus derived useful guidelines for the rational synthesis of more potent inhibitors. All compounds were docked into the catalytic site of human PDE3A using the FlexX module as implemented

Table 3. Inhibitory Effect of Compounds **5f**, **5u**, Milrinone, and Cilostazol on the Activity of PDE3 Isolated from Human Platelets[a]



compound	IC_{50} (μM)
5f	0.037
5u	0.078
milrinone	0.28
cilostazol	0.21

[a] Enzyme activity was measured as described in the Experimental Section.

in Sybyl. FlexX is a widely used docking algorithm in drug design. Its ability in predicting a conformation of the ligand very close to the one X-ray observed in its complex with the biological target has been fully described in the literature.[17]

According to the results of our calculations, milrinone and cilostazol, both characterized by a lactam function, bind in a similar way (Figure 2A), displaying two hydrogen bonds with the amino group of Lys947 side-chain and with the backbone carbonyl group of Thr908, respectively. In addition, in the case of milrinone, the cyano moiety protrudes toward His913 and Ile951 and the pyridine ring is closely packed to Tyr751 and His752, while for cilostazol, its 3,4-dihydroquinolin-2(1*H*)-one moiety shows extensive van der Waals interactions with both Leu910 and Ile951. The remainder of the molecule extends into the catalytic site with the butyl chain packed against Phe1004 and the cyclohexyl ring involved in a number of hydrophobic interactions with the side chains of Phe972, Phe989, and Leu1000 (Figure 2A).

In the case of our coumarin derivatives, showing a different chemical scaffold in comparison with the two reference structures, they occupy the same region of the catalytic core of the enzyme but involve different residues in the binding with PDE3A (Figure 2B). Both compounds **5f** and **5u** form two hydrogen bonds between their carbonyl group and the Nϵ atoms of His756 and His836 and the NH of their piperazine ring binds to the side-chain carbonyl group of Glu866. In addition, in the case of **5f**, the nitrogen of the pyridine ring is engaged with a further H-bond with the side-chain amino group of residue Gln1001, while for **5u**, this residue is involved in the binding with the morpholine oxygen. Previous results of some of us[18] highlighted, in the binding mode of cAMP with PDE3, two hydrogen bonds of the 6-amino group and the nitrogen at position 1 of the adenine moiety with the amide function of Gln1001. Therefore, it may be possible that, by exhibiting a polar interaction analogous to that of cAMP, **5f** and **5u** inhibit PDE3A in a better way than those ligands displaying different hydrophilic and/or hydrophobic interactions with the enzyme. Site-directed mutagenesis experiments could therefore help in carefully testing this assumption.

Besides these polar interactions, a number of hydrophobic interactions further stabilize the binding of compounds **5** inside

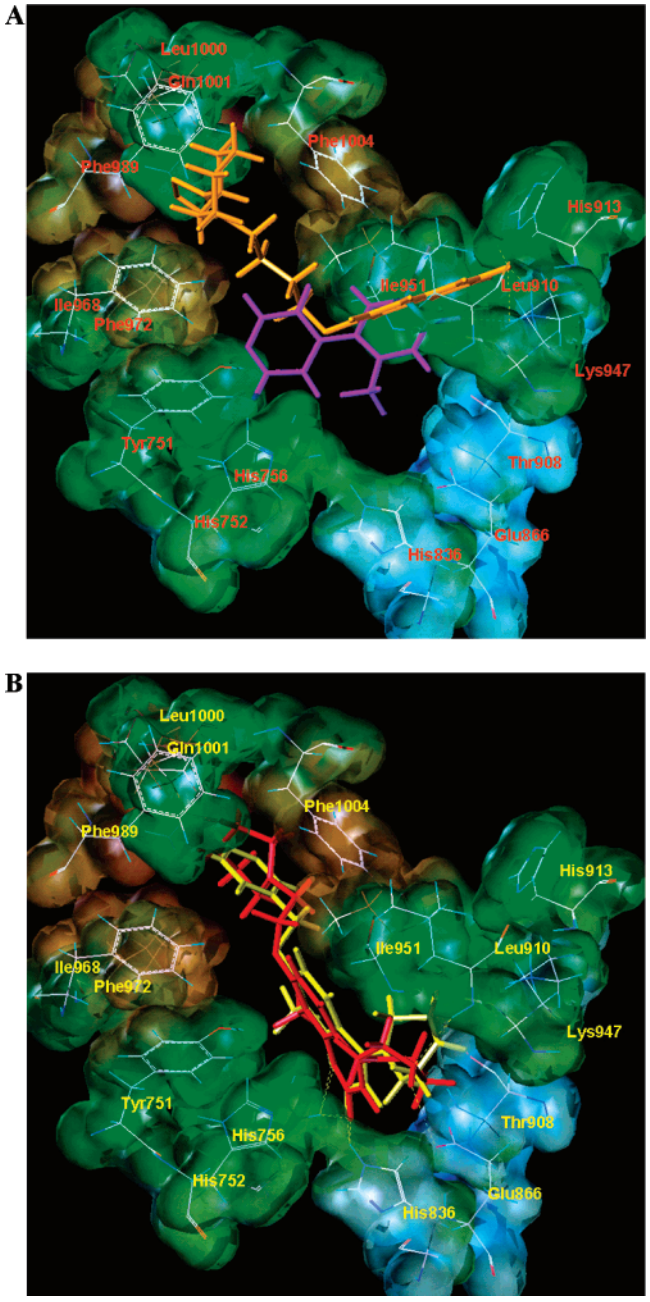


Figure 2. (A) Milrinone (violet) and cilostazol (orange) docked into the catalytic site of human PDE3A. For clarity, only the most important amino acid residues are reported. (B) Compounds **5f** (yellow) and **5u** (red) docked into the catalytic site of human PDE3A. For clarity, only the most important amino acid residues are reported.

the macromolecule, according to the results of our calculations. The two inhibitors both interact with Leu910 and Ile968, which surround the coumarinic scaffold, while the methyl on position 8 is oriented toward residue Ile951 occupying a small region delimited by residues Tyr751 and Ile951. This slight interaction could probably contribute to further anchoring the molecule into the catalytic site and is in agreement with experimental data because derivatives lacking the methyl on position 8 are less potent than the corresponding analogues 8-methyl-substituted (see Table 1, compounds **5d**, **5e**). Furthermore **5f**, showing a pyridine ring in its 7-alkoxy substituent, is able to perform a $\pi-\pi$ stacking with Phe1004. This latest interaction, performed only by cAMP, the natural substrate of the enzyme, and by the most potent inhibitors, could probably account for the higher affinity of **5f** compared to **5u** (see Table 3). These findings agree

with the site-directed mutagenesis experiments that have shown how Phe1004 mutations significantly impact the catalytic activity and nucleotide binding of PDE3A.[19]

Thus, the conclusions of our computational evaluation, still leaving some possibilities on the binding pose adopted by different inhibitors inside the catalytic pocket of the enzyme, further support our biological results and suggest that, while the inhibitory activity depends on a number of specific H-bond interactions anchoring the ligand to the counterpart, the potency is probably modulated by a larger number of hydrophobic interactions inside the catalytic site.

Experimental Section

Chemistry. Melting points were determined using a Fisher−Johns apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer "Spectrum One" spectrophotometer (abbreviations relative to IR bands: br = broad, s = strong, w = weak, sh = shoulder). ^{1}H NMR spectra were recorded on a Varian Gemini 200 (200 MHz) spectrometer and chemical shifts (δ) are reported in ppm using $(CH_3)_4Si$ as an internal reference ($\delta = 0$). Spin multiplicities are given as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Elemental analyses of all new compounds were performed at the Laboratorio di Microanalisi, Dipartimento di Scienze Farmaceutiche, Università di Genova, using a Carlo Erba Elemental Analyzer Model EA 1110, and all values were within ±0.4% of the calculated data.

Thin-layer chromatograms were run on Merck silica gel 60 F_{254} precoated plastic sheets (layer thickness 0.2 mm). Column chromatography was performed using Carlo Erba silica gel (0.05−0.20 mm).

General Procedure for the Synthesis of Substituted Acetophenones 18a−g,i−n. A mixture of 1-(2,5-dihydroxyphenyl)-ethanone **17a** (3.80 g, 25.0 mmol), 1-(2,4-dihydroxyphenyl)ethanone **17b** (3.80 g, 25.0 mmol), or 1-(2,4-dihydroxy-3-methylphenyl)-ethanone **17c** (4.15 g, 25.0 mmol), 25.0 mmol of the proper alkyl halide, 1.50 g of finely powdered KOH mixed with 4.0 g of anhydrous K_2CO_3 and 2-butanone (150 mL) was heated at reflux for 5 h (compounds **18c**, **18n**) or 16 h (compounds **18a**,**b**,**d**−**g**,**i**−**m**) with stirring. The mixture was then poured into cold water (500 mL): only in the case of compound **18n**, the crude compound separated out as a solid that was filtered, washed with water, and dried. In all other cases, an emulsion was obtained that was exhaustively extracted with diethyl ether (compounds **18d**−**g**) or dichloromethane (compounds **18a**−**c**,**i**−**m**). From the combined extracts (washed with water and dried over anhydrous Na_2SO_4), after removal of solvents under reduced pressure, an oily residue was obtained. Both the crude compound **18n** and the oily residues obtained by extraction were then chromatographed on a silica gel column to recover pure compound **18**. The elution was carried out with dichloromethane−petroleum ether (1:1) in the case of compounds **18d**−**g**,**n**, whereas in the case of compounds **18a**−**c**,**i**−**m**, their elution was performed with ethyl acetate−acetone (4:1) after having discarded some impurities previously eluted with dichloromethane. The pure compounds **18** were finally crystallized (compounds **18a**−**g**,**l**−**n**) or transformed into hydrochlorides and then crystallized (compounds **18i**−**k**). According to this procedure, compounds **18a**−**g**,**i**−**n** were prepared (yields: 32−85%).

Procedure for the Synthesis of Substituted Acetophenones 18h,o. A mixture of 1-(2,4-dihydroxy-3-methylphenyl)ethanone **17c** (4.15 g, 25.0 mmol), anhydrous K_2CO_3 (4.0 g), and the proper alkyl halide [3-(chloromethyl)-1-methylpiperidine hydrochloride (4.60 g, 25.0 mmol) in the presence of 10 mL of Dowtherm A in the case of **18h** or 5 mL of (2-chloroethoxy)benzene (used as solvent) in the case of **18o**] was heated at 160 °C for 16 h with stirring. In both cases, the resulting slurry was partitioned between water (150 mL) and 150 mL of a proper solvent (dichloromethane for **18h** and diethyl ether for **18o**). The organic phases were collected, and the aqueous ones were further extracted twice with the respective solvents. The combined extracts (dried over anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure) afforded oily residues that were purified through column chromatography. In the case of **18h**, the elution was first carried out with dichloromethane−ethyl acetate (1:1) to remove Dowtherm A and small amounts of the starting compound, then with ethyl acetate−acetone (1:1) to give pure **18h** (53%). In the case of **18o**, after having discarded the first fractions containing the excess (2-chloroethoxy)benzene by elution with dichloromethane−petroleum ether (1:1), the pure compound (30%) was obtained using the same eluant. Compounds **18h**,**o** were then crystallized.

General Procedure for the Synthesis of Substituted 4-Hydroxy-2*H*-1-benzopyran-2-ones 19a−o. A mixture of the proper substituted acetophenone **18** (10.0 mmol), sodium (0.58 g, 25.0 mmol), diethyl carbonate (3.54 g, 30.0 mmol), and Dowtherm A (10 mL) was heated at 160 °C with stirring. Within a few minutes, after the sodium melting, a sudden reaction occurred with formation of an abundant whitish precipitate. Xylene (30 mL) was added to dilute the mixture and allow the stirring, then the mixture was further heated at 160 °C for 1 h. After cooling, diethyl ether (30 mL) was added and the solid (crude compound **19** as sodium salt) was collected by filtration, washed with abundant diethyl ether, and dried. This solid was generally dissolved in water (50 mL), and the alkaline aqueous solution was acidified with 6 N aqueous HCl (compounds **19d**−**g**,**n**,**o**) or carefully acidified with 6 N aqueous HCl down to pH 4 (pyridylmethoxy derivatives **19a**−**c**) so that the compound **19** separated out as amorphous solid, which was collected by filtration, washed with a little water, and dried. In the case of compounds **19h**−**m** (zwitterionic structure), the crude sodium salt was dissolved/suspended in a very small amount of water (10 mL) and 6 N aqueous HCl was carefully added down to pH 4. The crude compounds **19h**−**m** that precipitated (**19i**,**j**,**k**,**m** as hydrochloride salts) after stirring for 30 min at room temperature, were collected by filtration, avoiding the washing with water, and finally dried. All crude compounds **19a**−**o** (**19i**,**j**,**k**,**m** as hydrochloride salts) were then crystallized (yields: 50−91%).

General Procedure for the Synthesis of Substituted 4-(1-Piperazinyl)-2*H*-1-benzopyran-2-ones 5i−u,w. A mixture of the proper compound **19** (3.0 mmol) and excess piperazine (7.0 g) was stirred at 160 °C for 1 h, then the final hot solution was poured into ice water. The resulting solution was exhaustively extracted with chloroform. The combined extracts (dried over anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure) afforded a thick oil from which (in the case of compounds **5i**,**l**,**q**,**t**,**u**,**w**), after treatment with diethyl ether and ethyl acetate and standing, nearly pure compound **5** separated out as a whitish solid that was then crystallized from the suitable solvent. In all the other cases (compounds **5j**,**k**,**m**−**p**,**r**,**s**), the final oily residue was treated with a solution of maleic acid (0.41 g, 3.5 mmol) in anhydrous ethanol to give the corresponding pure maleates (dimaleate in the case of **5j**) as white solids that were then crystallized from anhydrous ethanol. Compounds **5j**,**m**−**o** were subjected to biological assay as maleates, whereas the free amines **5k**,**p**,**r**,**s** were obtained (treatment of maleates with aqueous sodium bicarbonate and exhaustive extraction with chloroform) as thick oils that were then crystallized from the proper solvents to give white or whitish crystalline solids. Also, in the case of compounds **5j**,**m**−**o**, a little amount of the free base was obtained in a similar manner to record the IR and ^{1}H NMR spectra. Only the IR and ^{1}H NMR spectral data of compounds **5m** and **5u**, chosen as examples, are reported below.

4-(1-Piperazinyl)-6-(3-pyridylmethoxy)-2*H*-1-benzopyran-2-one (5i). Compound **5i** was obtained (0.49 g, 48%) from **19a** (0.81 g): yellowish crystals, mp 155−156 °C (ethyl acetate). Anal. ($C_{19}H_{19}N_3O_3$) C, H, N.

4-(1-Piperazinyl)-7-(3-pyridylmethoxy)-2*H*-1-benzopyran-2-one (5j). Compound **5j** was obtained as dimaleate (**5j**·$2C_4H_4O_4$; 0.99 g, 58%) from **19b** (0.81 g): whitish crystals, mp 159−161 °C (anhydrous ethanol). Anal. ($C_{19}H_{19}N_3O_3$·$2C_4H_4O_4$) C, H, N.

7-[(4-Methoxy-3,5-dimethyl-2-pyridyl)methoxy]-8-methyl-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5k). Compound **5k** was

obtained as **5k**·H_2O (0.56 g, 44%) from **19c** (1.02 g): whitish crystals, mp 150−152 °C (ethyl acetate). Anal. ($C_{23}H_{27}N_3O_4$·H_2O) C, H, N.

7-(Methoxymethoxy)-8-methyl-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5l). Compound **5l** was obtained (0.34 g, 37%) from **19d** (0.71 g): white crystals, mp 146−147 °C (ethyl acetate). Anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

8-Methyl-7-[(methylthio)methoxy]-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5m). Compound **5m** was obtained as maleate (**5m**·$C_4H_4O_4$; 0.43 g, 33%) from **19e** (0.76 g): whitish crystals, mp 178−179 °C (anhydrous ethanol). The IR and ^{1}H NMR spectra were recorded on the free base (whitish solid, mp 132−133 °C). IR (KBr): 3346 (w, NH), 1698 (s, CO) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.26 (s, 3H, SCH_3), 2.31 (s, 3H, 8-CH_3), 2.44 (s, 1H, NH; disappeared with D_2O), 2.98−3.30 (m, 8H, piperazine CH_2's), 5.25 (s, 2H, OCH_2SCH_3), 5.63 (s, 1H, H-3), 6.83 (d of AB q, J = 9 Hz, 1H, H-6), 7.42 (d of AB q, J = 9 Hz, 1H, H-5). Anal. ($C_{16}H_{20}N_2O_3S$·$C_4H_4O_4$) C, H, N, S.

7-(Ethoxymethoxy)-8-methyl-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5n). Compound **5n** was obtained as maleate (**5n**·$C_4H_4O_4$; 0.52 g, 40%) from **19f** (0.75 g): whitish crystals, mp 161−162 °C (anhydrous ethanol). Anal. ($C_{17}H_{22}N_2O_4$·$C_4H_4O_4$) C, H, N.

8-Methyl-7-[2-(methylthio)ethoxy]-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5o). Compound **5o** was obtained as maleate (**5o**·$C_4H_4O_4$; 0.60 g, 44%) from **19g** (0.80 g): whitish crystals, mp 173−174.5 °C (anhydrous ethanol). Anal. ($C_{17}H_{22}N_2O_3S$·$C_4H_4O_4$) C, H, N, S.

8-Methyl-7-[(1-methyl-3-piperidinyl)methoxy]-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5p). Compound **5p** was obtained (0.45 g, 40%) from **19h** (0.94 g): whitish crystals, mp 158−160 °C (ethyl acetate/petroleum ether). Anal. ($C_{21}H_{29}N_3O_3$) C, H, N.

7-[2-(Dimethylamino)ethoxy]-8-methyl-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5q). Compound **5q** was obtained (0.48 g, 48%) from **19i**·HCl (0.90 g): ivory-white crystals, mp 138−139.5 °C (ethyl acetate/petroleum ether). Anal. ($C_{18}H_{25}N_3O_3$) C, H, N.

7-[2-(Diethylamino)ethoxy]-8-methyl-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5r). Compound **5r** was obtained (0.51 g, 47%) from **19j**·HCl (0.98 g): whitish crystals, mp 110−111 °C (ethyl acetate/diethyl ether). Anal. ($C_{20}H_{29}N_3O_3$) C, H, N.

8-Methyl-4-(1-piperazinyl)-7-[2-(1-pyrrolidinyl)ethoxy]-2*H*-1-benzopyran-2-one (5s). Compound **5s** was obtained (0.74 g, 69%) from **19k**·HCl·0.5H_2O (1.00 g): whitish crystals, mp 142−143 °C (ethyl acetate). Anal. ($C_{20}H_{27}N_3O_3$) C, H, N.

8-Methyl-4-(1-piperazinyl)-7-[2-(1-piperidinyl)ethoxy]-2*H*-1-benzopyran-2-one (5t). Compound **5t** was obtained (0.45 g, 40%) from **19l**·H_2O (0.96 g): ivory-white crystals, mp 146−147 °C (ethyl acetate). Anal. ($C_{21}H_{29}N_3O_3$) C, H, N.

8-Methyl-7-(2-morpholinoethoxy)-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5u). Compound **5u** was obtained (0.56 g, 50%) from **19m**·HCl·0.5H_2O (1.05 g): ivory-white crystals, mp 180−181 °C (ethyl acetate). IR (KBr): 3348 (w, NH), 1702 (s, CO) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.18 (s, 1H, NH; disappeared with D_2O), 2.31 (s, 3H, 8-CH_3), 2.63 (near t, 4H, morpholine NCH_2's), 2.88 (t, 2H, OCH_2CH_2N), 3.06−3.34 (m, 8H, piperazine CH_2's), 3.75 (near t, 4H, morpholine OCH_2's), 4.21 (t, 2H, OCH_2-CH_2N), 5.63 (s, 1H, H-3), 6.80 (d of AB q, J = 9 Hz, 1H, H-6), 7.43 (d of AB q, J = 9 Hz, 1H, H-5). Anal. ($C_{20}H_{27}N_3O_4$) C, H, N.

8-Methyl-7-(2-phenoxyethoxy)-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5w). Compound **5w** was obtained (0.34 g, 30%) from **19o** (0.94 g): whitish crystals, mp 169−170 °C (ethyl acetate/petroleum ether). Anal. ($C_{22}H_{24}N_2O_4$) C, H, N.

4-Chloro-8-methyl-7-(2-phenylbenzyloxy)-2*H*-1-benzopyran-2-one (20a). A mixture of **19n** (1.08 g, 3.0 mmol), triethylamine (0.30 g, 3.0 mmol), and phosphorus oxychloride (3.0 mL) was stirred at 120 °C for 30 min. The final reaction mixture was poured into ice water, stirred, and then exhaustively extracted with dichloromethane. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure to afford a dark reddish oil that was chromatographed on a silica gel column eluting with the mixture dichloromethane−petroleum ether (2:1) to give the nearly pure **20a** (0.60 g, 53%): white crystals, mp 151−152 °C (diisopropyl ether). Anal. ($C_{23}H_{17}ClO_3$) C, H.

8-Methyl-7-(2-phenylbenzyloxy)-4-(1-piperazinyl)-2*H*-1-benzopyran-2-one (5v). A mixture of chloro derivative **20a** (0.75 g, 2.0 mmol), piperazine (1.72 g, 20.0 mmol), and ethanol (150 mL) was refluxed for 1 h with stirring. The solvent was then removed and the residue was partitioned between water and chloroform; the organic phase was collected and the aqueous one was further extracted twice with chloroform. The combined extracts (dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure) afforded compound **5v** (0.78 g, 85%): white crystals, mp 208−209 °C (ethyl acetate). Anal. ($C_{27}H_{26}N_2O_3$) C, H, N.

4-(4-Amino-1-piperidinyl)-8-methyl-7-(3-pyridylmethoxy)-2*H*-1-benzopyran-2-one (5x). A mixture of 4-chloro-8-methyl-7-(3-pyridylmethoxy)-2*H*-1-benzopyran-2-one **20b**[8] (0.60 g, 2.0 mmol), 4-aminopiperidine (2.00 g, 20.0 mmol), and ethanol (150 mL) was stirred at room temperature for 60 h. The solvent was then removed, and the residue was partitioned between water and chloroform; the organic phase was collected and the aqueous one was further extracted twice with chloroform. The combined extracts (dried over anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure) gave a solid residue that was chromatographed on a silica gel column, eluting with methanol to remove some impurities, then with the mixture methanol−triethylamine (9:1) to afford compound **5x** (0.49 g, 68%): white crystals, mp 186−187 °C (dichloromethane/petroleum ether). Anal. ($C_{21}H_{23}N_3O_3$) C, H, N.

Preparation of Quinoline Derivatives 22a,b. A mixture of the proper chloro derivative **21a**[15] (0.39 g, 2.0 mmol) or **21b**[16] (0.39 g, 2.0 mmol) and piperazine (1.72 g, 20.0 mmol) was refluxed under stirring for 3 h in ethanol (150 mL, in the case of the preparation of **22a**) or heated at 150 °C for 1 h in ethylene glycol (10 mL, in the case of preparation of **22b**). In the first case, the solvent was removed under reduced pressure and the residue was partitioned between water and chloroform; in the second case, the final mixture was poured into ice water and extracted with chloroform. In both cases, after an exhaustive extraction with chloroform, the combined organic phases were dried over anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure to afford a residue from which compounds **22a** or **22b** separated out, after treatment with the proper solvent, as crystalline solids.

1-Methyl-4-(1-piperazinyl)quinolin-2(1*H*)-one (22a). Compound **22a** was obtained as a white solid (0.44 g, 90%) by treating with isopropyl ether the oily residue derived from the reaction carried out with **21a**: white crystals, mp 122−123 °C (ethyl acetate/petroleum ether). Anal. ($C_{14}H_{17}N_3O$) C, H, N.

8-Methyl-4-(1-piperazinyl)quinolin-2(1*H*)-one (22b). Compound **22b** was obtained as a whitish solid (0.38 g, 78%) by treating with ethyl acetate the resinous residue derived from the reaction carried out with **21b**: white crystals, mp 233−234 °C (dichloromethane/ethyl acetate). Anal. ($C_{14}H_{17}N_3O$) C, H, N.

Biological Methods. Evaluation of Antiplatelet Activity in Human PRP. Human blood from healthy volunteers was added to a trisodium citrate 130 mM aqueous solution (volume ratio 9:1) and then centrifuged at 100 *g* for 30 min to give PRP, which was diluted to 2.0×10^8 plts/mL with platelet-poor plasma. The diluted PRP (500 μL) was preincubated at 37 °C for 2 min with solvent (dimethylsulfoxide, 5 μL) or drug solution (5 μL) before the addition of the platelet aggregation agent. PRP aggregation was induced by ADP (Sigma), collagen from bovine tendon (Mascia Brunelli), or A23187 (Sigma), at the 5.0 μM, 5.0 μg/mL, or 20.0 μM final concentrations, respectively. Before each experiment, the stock solutions of ADP (saline), collagen (saline), and A23187 (DMSO) were diluted in saline. Platelet aggregation, performed in an Aggrecoder PA-3210 aggregometer (A. Menarini, Florence, Italy), was measured following the Born's turbidimetric method[20] and quantified by the light transmission reached after 3 min.

To calculate the percentage of inhibition, the extent of aggregation measured in the presence of the compounds tested was always compared with that measured for a control sample containing the solvent, in an experiment carried out under the same conditions.

From each series of experiments, in which the inhibitors were tested in at least five concentrations, a percentage inhibition−concentration curve was derived. From this curve, the IC_{50} value was calculated as the concentration of inhibitor causing a 50% inhibition of the aggregation.

Statistical Analysis. The IC_{50} values reported in Table 2 are averages (±standard deviation) of those obtained from at least four independent determinations carried out on different batches of platelets (usually 5−8 batches). Statistical analysis was performed using the Student's *t*-test, considering significant the activity difference between two compounds when corresponding to $P < 0.05$.

PDE3 Activity Assay. Platelet soluble cyclic nucleotide PDE3 was purified as in Weishaar et al.[21] The enzyme activity was tested on PDE3 isolated from human platelets according to Thompson and Strada[22] with minor modifications. Briefly, phosphodiesterase activity was assayed after 5 min of preincubation at 30 °C of suitable aliquots of the purified enzyme both in the solvent and in the solutions containing the agents. The reaction started by the addition of the substrate (0.1 μCi/μL [^{3}H]cAMP and 0.1 μM cAMP). The incubation, prolonged for 30 min at 30 °C, was stopped by boiling samples for 90 s. The cooled mixtures were added to 25 μg/mL 5′-nucleotidase (5′-ribonucleotide phosphohydrolase from *Crotalus atrox* venom: EC 3.1.3.5) and incubated for additional 15 min at 30 °C. The [^{3}H]adenosine formed was eluted with 50% methanol from 2 mL AG1 × 8 resin (200−400 mesh, BIO-RAD pre-packed column). The eluates were collected in scintillation vials, and the radioactivity was counted in a Beckman LS6500 liquid scintillation counter. The values of IC_{50} (concentration that inhibits substrate hydrolysis by 50%) were determined from dose−response curves in which the concentrations of agents ranged from 10^{-9} M to 10^{-5} M for the more potent inhibitors and from 10^{-8} M to 10^{-4} M for the less potent ones.

Molecular Modeling. Molecular structures of ligands **5f**, **5u**, milrinone, and cilostazol were built and energy minimized within MacroModel (MacroModel 7.0, Schrödinger LLC).[23] Conformational analysis was carried out using the AMBER* force field, as included in MacroModel. For all compounds, the resulting geometries of the lowest energy conformers were re-optimized with semiempirical quantum mechanic calculations, using the Hamiltonian AM1, as implemented in Spartan (Spartan '02, Wavefunction Inc., Irvine, CA.).

The three-dimensional model of PDE3A, already published by some of us,[18] was used for these docking studies. Each inhibitor was docked into the active site of the enzyme by means of the FlexX module, as implemented in Sybyl 7.1 (Sybyl 7.1, Tripos Inc., 1699 South Hanley Road, St. Louis, Missouri, 63144, U.S.A.), which keeps the macromolecule and the ligands flexible. Because the preparation of the protein for FlexX requires definition of the binding pocket in terms of "interaction points", the active site was defined as all atoms located within a distance of 10 Å from residues His752, Ile951, Ile968, Phe972, Leu1000, and Phe1004, which, according to the results of our previous study,[18] interact with cAMP, the natural substrate of the enzyme. This specific distance was determined to ensure that a significant portion of the catalytic site was available for the docking experiments. The results were evaluated in terms of total estimated binding energy, internal strain energy of the ligand, and van der Waals and electrostatic interaction energies. All calculations were carried out on a SGI O2 workstation and on a standard personal computer running under Linux.

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Supporting Information Available: Elemental analysis data of all new compounds described; experimental details of synthetic procedures for compounds **18a**−**o** and **19a**−**o**; IR and ^{1}H NMR data of substituted acetophenone **18m** (chosen as an example) and of substituted 4-hydroxycoumarins **19a**−**o**; IR and ^{1}H NMR data of substituted 4-(1-piperazinyl)coumarins **5i**−**l**,**n**−**t**,**v**,**w**, and their analogue **5x**, 4-chlorocoumarin derivative **20b**, and quinoline derivatives **22a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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