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Analysis of anti-PDE3 activity of 2-morpholinochromone derivatives reveals multiple mechanisms of anti-platelet activity

Belinda M. Abbott[a,†] and Philip E. Thompson[b,*]

[a]Department of Medicine, Monash University, Box Hill Hospital, Box Hill 3128, Australia
[b]Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University, Parkville 3052, Australia

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Abstract—A series of synthetic 2-morpholinochromones have been evaluated as inhibitors of platelet phosphodiesterase, PDE3A. While previous assertions about the anti-PDE3 activity of this class have been confirmed, in some cases the reported anti-platelet activities clearly derive from a non-PDE3 regulated mechanism. The potential utility of 2-morpholinochromones either as PDE3 inhibitors and/or anti-thrombotic agents thus remains only poorly examined.

The cyclic nucleotides, cAMP and cGMP, play important roles in cells as second messenger molecules, activating and co-ordinating intracellular activity of an array of proteins. The regulation of cyclic nucleotides by phosphodiesterases is very complex with most cells utilising multiple PDE isoforms to effect hydrolysis.[1,2] These multiple isoforms have varied expression, subcellular compartmentalisation and other means of activation or inhibition, including cross-isoform communication, with cGMP and cAMP competing for catalytic and allosteric sites on specific isoforms, which determine the mode and extent of nucleotide hydrolysis. Phosphodiesterases (PDEs) therefore effectively distribute cyclic nucleotides around the cell and regulate downstream activation of specific protein kinases and so can be considered active contributors to cell signalling pathways.

Subclassification of PDEs was originally established according to the substrate preferences and the effect of certain pharmacological inhibitors. The isozyme now known as PDE3 was first characterised as 'low-K_m, cGMP-inhibited, cAMP phosphodiesterase'. That pharmacological classification still holds up today, but with additional refinements relating to recognition of homologous but distinct gene products and splice variants within those classes. The PDE3 family consists of two distinct gene products, PDE3A and PDE3B, from chromosomes 12 and 11, respectively.[3]

Early studies suggested that PDE3 inhibitors such as milrinone, enoximone and cilostazol (Fig. 1) showed positive inotropic and anti-platelet effects which could have utility in the treatment of heart failure and thrombosis, respectively. However, adverse outcomes in early clinical studies hampered progress in this area.[4] More recently, re-analysis of clinical data suggests that improved dosing regimes may improve the therapeutic window of PDE3 inhibitors against heart failure.[5] The FDA approval of Pletal® (cilostazol) for use in peripheral artery occlusive disease (PAOD) is showing the utility of PDE3 inhibitors in an anti-thrombotic context.[6] Phosphodiesterase inhibition has re-emerged as a promising strategy for treating many diseases, as improved pharmacological studies dissect the fine requirements for therapeutic efficacy, including isozyme selectivity.[7–9]

The study of synthetic 2-morpholinochromones has yielded a number of bioactive molecules in the past two decades (Fig. 2). The most notable member of this chemical class is LY294002, a selective phosphatidylinositol 3-kinase inhibitor,[10,11] which has been widely used

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* Corresponding author. Tel.: +613 9903 9672; fax: +613 9903 9582; e-mail: phil.thompson@vcp.monash.edu.au
† Present address: Department of Biological Chemistry and Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee DD1 4HN, Scotland.

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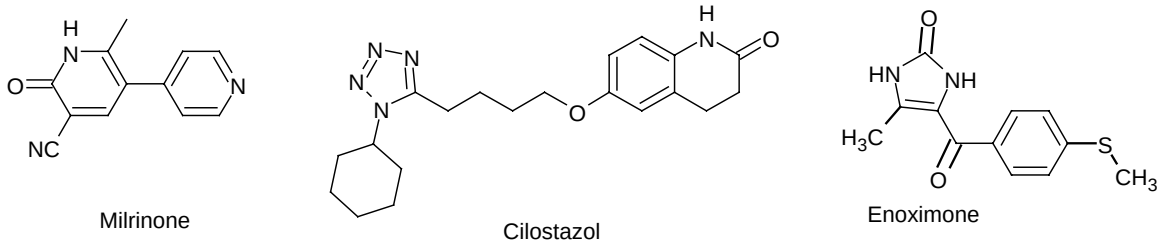


Figure 1. Structures of known inhibitors of PDE3.

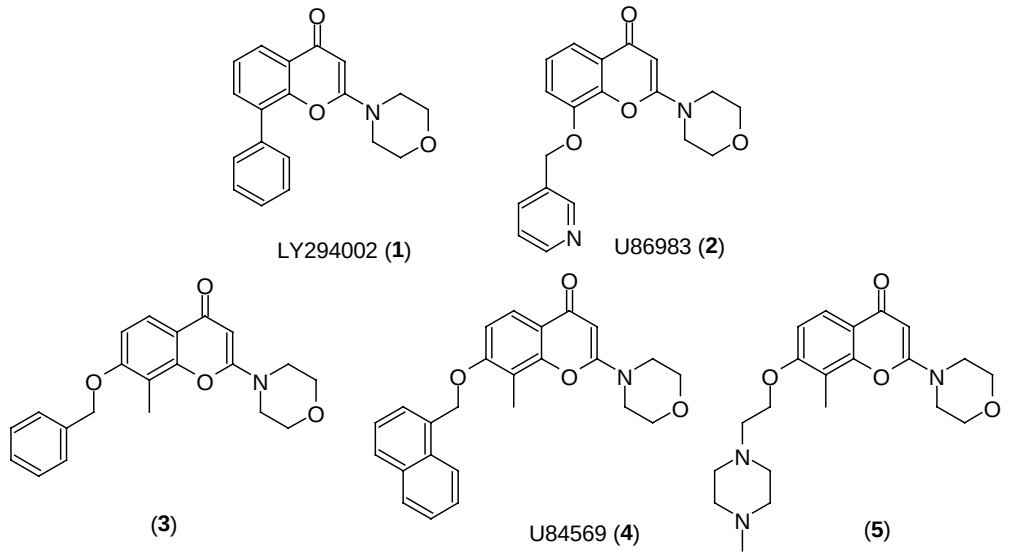


Figure 2. Structures of reported 2-morpholinochromones.

in the characterisation of that enzymes' cellular functions including those in platelets.[12] Other synthetic 2-morpholinochromones described with biological activity include U86983, an inhibitor of cell proliferation and migration, and inhibitors of platelet aggregation.[13] U84569 (**4**) was one of an array of analogues of compound **3** that were prepared and the anti-platelet activity of U84569 was determined to be due to inhibition of PDE3 in platelets. Later, compound **5** was reported as a potent inhibitor of platelet aggregation that showed anti-thrombotic activity in vivo in the Folts' canine model of thrombosis.[14] By analogy with studies of U84569, inhibition of PDE3 was proposed as the mechanism of action. In addition, Leoncini et al. have proposed this mechanism of action for the anti-platelet activity of other 2-aminochromones and a host of analogues.[15,16]

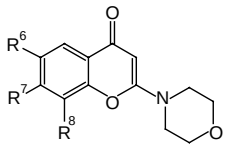
In an earlier report, we showed that LY294002 was only a poor inhibitor of PDE3A with an IC_{50} of approximately 100 μM, but had significant activity against the PDE2 isoform.[17] The array of activities shown for these compounds against platelet enzymes other than PDE3A suggested that other mechanisms of anti-platelet activity might have confounded attempts to develop structure–activity relationships. We decided to perform a more systematic survey of the PDE3A inhibition of 2-morpholinochromones than had been previously described.

The library of 2-morpholinochromones tested is summarised in Table 1. The general synthetic routes used to prepare them were performed as described previously.[14,17,18] The aryl ethers, **13** and **18** were prepared by copper mediated coupling of the precursor phenols with phenylboronic acid, albeit in low yield (8% and 3%, respectively) according to the conditions outlined in Scheme 1.[19,20]

The PDE3A isozyme was purified from human platelet lysate by anion exchange chromatography according to the general procedure of Dickinson et al.[21] Activity of pooled fractions was determined by assays of AMP product formation quantified by reverse-phase HPLC.[22] The activity of the PDE3A isoform was confirmed by the use of the selective inhibitors cilostamide and milrinone.

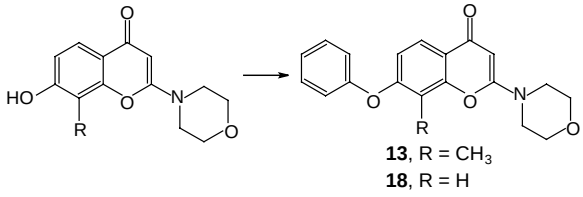
Synthetic 2-morpholinochromones of different structural classes were evaluated against isolated PDE3A in a screening assay at 50 μM. The results of this screen are shown in Table 1. The most potent inhibitors in this screen were all 8-methyl-7-substituted compounds, which reaffirmed the original postulate that members of this class of compounds are, in general, inhibitors of PDE3A. Strikingly however, the only inactive compound in this class was compound (**5**), a result apparently at odds with its reported anti-platelet potency. In

Table 1. Molecular structures and PDE3 inhibition of the 2-morpholinochromones



Compound	R^6	R^7	R^8	Ref.	% PDE3 inhibition[a]
1	H	H	Ph	11	35 ± 2
2	H	H	OCH_2(3-Pyridyl)	14	5 ± 5
3	H	OCH_2Ph	Me	13,14	86 ± 2
5	H	$(CH_2)_2$(4-Me-1-Piperazinyl)	Me	13	−10 ± 3
6	OMe	H	(4-Cl)Ph	17	63 ± 2
7	H	H	OCH_2Ph	14	−7, $n = 1$
8	H	H	C≡CPh	14	60 ± 1
9	H	H	$(CH_2)_2Ph$	14	28 ± 1
10	H	OCH_2(3-pyridyl)	Me	13,14	68 ± 4
11	H	Ph	Me	14	83 ± 2
12	H	C≡CPh	Me	14	44 ± 4
13	H	OPh	Me	[b]	78 ± 5
14	H	OCH_2Ph	H	14	49 ± 1
15	H	OCH_2(3-pyridyl)	H	14	19 ± 3
16	H	Ph	H	17	50 ± 5
17	H	C≡CPh	H	17	70 ± 1
18	H	OPh	H	[b]	43 ± 2

[a] (% basal) at 50 μM. All values are means ± SE ($n \geqslant 2$) except where indicated.
[b] See Scheme 1. HR-ESI-MS **13** *m/z* 338.1388 (M+H, $C_{20}H_{19}NO_4$ requires 338.1392); **18** *m/z* 324.1231 (M+H, $C_{19}H_{17}NO_4$ requires 324.1236).



Scheme 1. Reagents and conditions: $Cu(OAc)_2$, phenylboronic acid, triethylamine DCM, rt, 48 h.

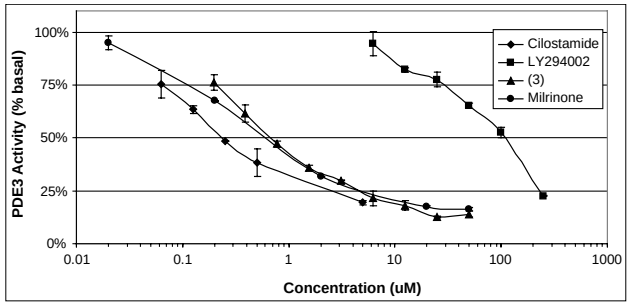


Figure 3. Inhibition of **3** compared with milrinone, cilostamide and LY294002 (**1**).

addition, compound (**10**) was found to be less active than (**3**), despite reportedly being a more potent inhibitor of platelet aggregation.

Inhibition of PDE3A was reduced to less than 50% in compounds where 8-methyl substitution was removed (compounds **14**–**16**, **18**). Similarly, compounds with substitution in position 8, such as LY294002 (**1**) and U-86983 (**2**), showed relatively poor ability to inhibit PDE3A. The exception to these generalisations was the alkynyl compounds, with phenylethynyl compounds (**8**) and (**17**), both more potent than the 8-methyl counterpart (**12**). It should be noted that these two compounds also inhibit PDE2 quite strongly.[17] The results from the preliminary screen prompted us to give attention to the most potent compound of the series, (**3**). PDE3A inhibition was demonstrated in a dose-dependent manner (Fig. 3), and was equipotent with milrinone in this assay, with an IC_{50} of 0.6 μM.

While our results showed that compound (**5**) possessed no anti-PDE3 activity, we were able to confirm that it potently inhibited ADP-induced aggregation of platelet rich plasma (PRP), as reported.[14] Moreover, the potency of other compounds (**3**) and (**10**) was also consistent with those reported data. As yet we have no explanation for the mechanism of action, but we have been able to rule out inhibition of PI3 kinase and PDE2 activity, which are similarly refractory to treatment with **5**. One telling observation is that the anti-platelet activity is *not* preserved if aggregations are performed using washed platelet suspensions,[23] with concentrations of 100 μM insufficient to block aggregation by the platelet agonists, PAR4AP (Fig. 4), TRAP and collagen related peptide (CRP) under those conditions, while compound (**3**) at 25 μM completely abolished aggregation consistent with its PDE3 inhibitory potency (Fig. 4). The implication is that the anti-platelet activity of **5** is dependent upon some factor from plasma.

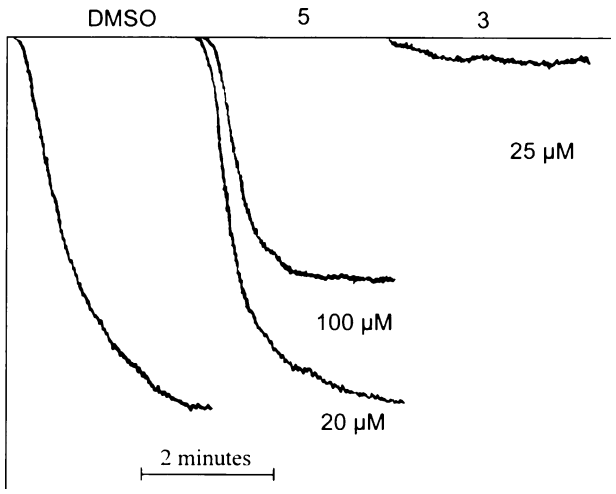


Figure 4. Inhibition of PAR4AP-induced washed platelet aggregation by **3** (25 μM) and **5** (20 and 100 μM) in comparison with DMSO (vehicle).

Interestingly, compound **10** showed intermediate activity between that observed for **3** and **5** in both platelet assays and PDE3 inhibition assays ($46 \pm 4\%$ inhibition at 6.25 μM), suggestive of a combination of the observed anti-PDE3 activity and the other yet-to-be-characterised activity. A comparable activity was shown by the recently described 4-(1-piperazinyl)coumarin, RC-414, which has a very similar substituted structure (Fig. 5).[16] RC-414 also shows a relatively enhanced anti-platelet activity in PRP compared to washed platelet suspensions, and its activity has been speculated to relate to combined PDE3 inhibition and increased nitric oxide formation. It is tempting to suggest that the same additional mechanism may apply in the case of **5** and **10**.

In summary, these results reveal new features of the anti-platelet effects of 2-morpholinochromones, including multiple mechanisms of action that mean the anti-PDE3 activity of these compounds and the biological consequences of that inhibition remaining largely unexplored. Our general screen of inhibitors has given us some indicators of features desirable to maintain PDE3A activity. In particular, the C8 methyl function with a C7 aryl moiety appears to give the most potent inhibitors. This profile is also distinct from that typical of PDE2, PI3 kinase or DNA-dependent protein kinase[24] for which 2-morpholinochromones have recently been assessed.

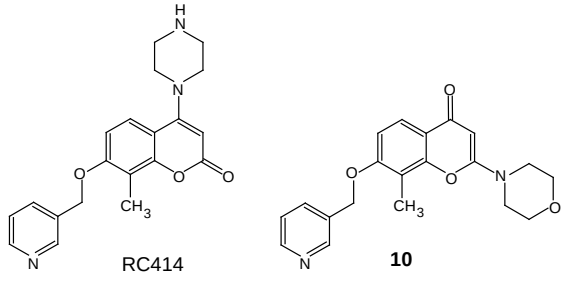


Figure 5. Structures of RC-414 and **10**.

In addition, renewed analysis of the mechanism by which compound **5** exerts its effects is warranted. This compound has been shown to have efficacy in vivo in a canine model of thrombosis, with significant separation from hemodynamic effects.[14] Determination of the mechanism might generate a useful new path to novel anti-thrombotic agents.

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References and notes

1. Manganiello, V. C.; Degerman, E. *Thromb. Haemost.* **1999**, *82*, 407.
2. Beavo, J. A.; Brunton, L. L. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 710.
3. Degerman, E.; Belfrage, P.; Manganiello, V. C. *J. Biol. Chem.* **1997**, *272*, 6823.
4. Packer, M.; Carver, J. R.; Rodeheffer, R. J.; Ivanhoe, R. J.; DiBianco, R.; Zeldis, S. M.; Hendrix, G. H.; Bommer, W. J.; Elkayam, U.; Kukin, M. L., et al. *N. Engl. J. Med.* **1991**, *325*, 1468.
5. Shakar, S. F.; Abraham, W. T.; Gilbert, E. M.; Robertson, A. D.; Lowes, B. D.; Zisman, L. S.; Ferguson, D. A.; Bristow, M. R. *J. Am. Coll. Cardiol.* **1998**, *31*, 1336.
6. Ikeda, Y. *Thromb. Haemost.* **1999**, *82*, 435.
7. Manallack, D.; Hughes, R. A.; Thompson, P. E. *J. Med. Chem.* **2005**, *48*, 3449.
8. Degerman, E. In *Diabetes Mellitus: A Fundamental and Clinical Text*; Le Roith, D., Olefsky, J. M., Taylor, S., Eds.; Lippincott: Philadelphia, 2004; p 373.
9. Edmondson, S. D.; Mastracchio, A.; He, J.; Chung, C. C.; Forrest, M. J.; Hofsess, S.; MacIntyre, E.; Metzger, J.; O'Connor, N.; Patel, K.; Tong, X.; Tota, M. R.; Van der Ploeg, L. H.; Varnerin, J. P.; Fisher, M. H.; Wyvratt, M. J.; Weber, A. E.; Parmee, E. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3983.
10. Vlahos, C. J.; Matter, W. F.; Hui, K. Y.; Brown, R. F. *J. Biol. Chem.* **1994**, *269*, 5241.
11. Abbott, B.; Thompson, P. *Aust. J. Chem.* **2003**, *56*, 1099.
12. Jackson, S. P.; Schoenwaelder, S. M.; Goncalves, I.; Nesbitt, W. S.; Yap, C. L.; Wright, C. E.; Kenche, V.; Anderson, K. E.; Dopheide, S. M.; Yuan, Y.; Sturgeon, S. A.; Prabaharan, H.; Thompson, P. E.; Smith, G. D.; Shepherd, P. R.; Daniele, N.; Kulkarni, S.; Abbott, B.; Saylik, D.; Jones, C.; Lu, L.; Giuliano, S.; Hughan, S. C.; Angus, J. A.; Robertson, A. D.; Salem, H. H. *Nat. Med.* **2005**, *11*, 507.
13. Benjamin, C. W.; Lin, A. H.; Morris, J.; Wishka, D. G.; Gorman, R. R. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 457.
14. Morris, J.; Wishka, D. G.; Lin, A. H.; Humphrey, W. R.; Wiltse, A. L.; Gammill, R. B.; Judge, T. M.; Bisaha, S. N.; Olds, N. L.; Jacob, C. S., et al. *J. Med. Chem.* **1993**, *36*, 2026.
15. Leoncini, G.; Maresca, M.; Colao, C.; Buzzi, E.; Mazzei, M.; Balbi, A. *Pharmacol. Res.* **1991**, *23*, 139.
16. Leoncini, G.; Signorello, M. G.; Bruzzese, D.; Di Braccio, M.; Grossi, G. C.; Roma, G. *Biochem. Pharmacol.* **2004**, *67*, 911.

17. Abbott, B. M.; Thompson, P. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2487.
18. Gammill, R. B.; Judge, T. M.; Morris, J. International Publication Number WO 91/19707, 1991.
19. Chan, D. T.; Monaco, K. L.; Wang, R.-P.; Winters, M. P. *Tetrahedron Lett.* **1998**, *39*, 2933.
20. Evans, D. A.; Katz, J. L.; West, T. R. *Tetrahedron Lett.* **1998**, *39*, 2937.
21. Dickinson, N. T.; Jang, E. K.; Haslam, R. J. *Biochem. J.* **1997**, *323*, 371.
22. Abbott, B. M.; Thompson, P. E. *Anal. Biochem.* **2005**, *339*, 185.
23. Washed platelet suspensions were prepared essentially according to the method of Schoenwaelder, S. M.; Yuan, Y.; Cooray, P.; Salem, H. H.; Jackson, S. P. *J. Biol. Chem.* **1997**, *272*, 1694. In brief, platelet rich plasma (PRP) from acid–citrate–dextrose (ACD)-treated whole blood was centrifuged at 2000*g* for 10 min and then resuspended in platelet wash buffer to 10% of the original volume. The washed platelets were then centrifuged at 4000*g* for 2 min, and the platelets were resuspended in Tyrode's buffer containing 1 mM $CaCl_2$. Aggregations were performed by treating the platelet suspensions (400 μL) with test compound or vehicle (DMSO, 1 μL) in the presence of 0.5 μg/mL fibrinogen. Agonists such as CRP, TRAP and PAR4AP were added at a concentration to give complete aggregation as earlier determined by dose–response titration. Aggregation was monitored over 10 min for each sample.
24. Griffin, R. J.; Fontana, G.; Golding, B. T.; Guiard, S.; Hardcastle, I. R.; Leahy, J. J.; Martin, N.; Richardson, C.; Rigoreau, L.; Stockley, M.; Smith, G. C. *J. Med. Chem.* **2005**, *48*, 569.