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PDE2 inhibition by the PI3 kinase inhibitor LY294002 and analogues

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Abstract—Synthetic 2-morpholinochromones, including the known PI3-kinase inhibitor LY294002, have been evaluated in vitro as inhibitors of isolated human platelet phosphodiesterases. Inhibition of the cAMP-phosphodiesterases, PDE2 and PDE3 by LY294002 is reported for the first time. Preliminary screening across a range of 2-morpholinochromones has revealed structural features for optimised PDE2 inhibition.

Cyclic nucleotide phosphodiesterases (PDEs) are important regulators of cellular functions, catalysing the hydrolysis and deactivation of the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).[1] The regulation of cyclic nucleotide levels by PDEs is very complex with most cells utilising multiple PDE isoforms to effect hydrolysis. The activities of the isoforms themselves are subject to regulation by varied expression levels, subcellular compartmentalisation, activation and inhibition. Activity is further subject to cross-isoform communication, with cGMP and cAMP competing for catalytic and allosteric sites on specific isoforms, which determines the mode and extent of nucleotide hydrolysis. In this way, PDEs are active contributors to cell signalling pathways, regulating intracellular distribution of cyclic nucleotides and therefore downstream activation of specific protein kinases.

The understanding of phosphodiesterases has been accelerated in recent years by the advances of molecular cloning and the concomitant development of isoform selective inhibitors,[2] most famously by the cGMP–PDE5 selective inhibitor, sildenafil, but also with cAMP–PDE3 inhibitors (e.g., cilostazol and milrinone) and cAMP–PDE4 inhibitors (e.g., rolipram). While further progress in these areas is fuelled by the pharmacological characterisation of these inhibitors, the understanding of those isoforms for which selective inhibitors are not known has tended to languish. For example, relatively little is known about PDE2, which is widely expressed in human tissues and hydrolyses both cAMP and cGMP. This activity may be significant as PDE2 has contrasting enzyme kinetics to other PDEs and is also allosterically activated by cGMP.[3] Thus, PDE2 may play a key role in regulating cyclic nucleotide levels under conditions where other PDEs are ineffective.

Progress in the pharmacological study of PDE2 has been hampered by the lack of a selective inhibitor. Only EHNA (*erythro*-9-(2-hydroxy-3-nonyl)adenine) (Fig. 1) has been described as exhibiting PDE2-isozyme

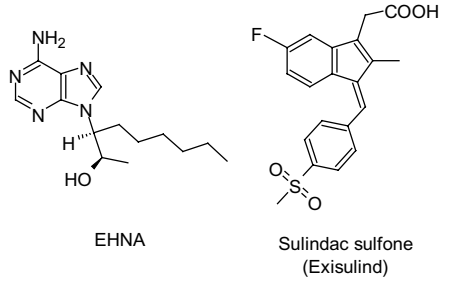

Figure 1. Structures of known inhibitors of PDE2.

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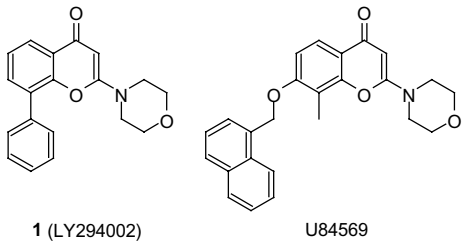


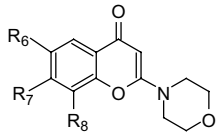
Figure 2. Structure of the 2-morpholinochromones LY294002 **1** and U84569.

selectivity with moderate potency.[4] However, EHNA is also a potent inhibitor of adenosine deaminase, an activity, which compromises its use in ascribing physiological roles to PDE2. A new contributor to our understanding of PDE2 function is the antitumour agent sulindac sulfone (exisulind) (Fig. 1), which appears to act by inhibition of both PDE5 and PDE2.[5] A number of sulindac sulfone and EHNA analogues have been the subject of recent patent applications covering diseases such as cancer, scleroderma and cognitive disorders.[6] However, the broader selectivity or otherwise of these analogues has not yet been described.

Our own interest in PDE2 began with our structure–function studies of the phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (**1**)[7] (Fig. 2). We were aware that other synthetic 2-morpholinochromones had been described as inhibitors of platelet cAMP phosphodiesterase, in particular PDE3 as reported for U84569[8] (Fig. 2), so we prepared a library of analogues with a view to studying the specificities of 2-morpholinochromones for PI3K and PDE3. As the procedure for assaying PDE3 activity demanded separation of PDE2 and PDE3 from platelet lysate,[9] we were also able to evaluate these compounds as potential inhibitors of PDE2. The surprising results of that study are presented here.

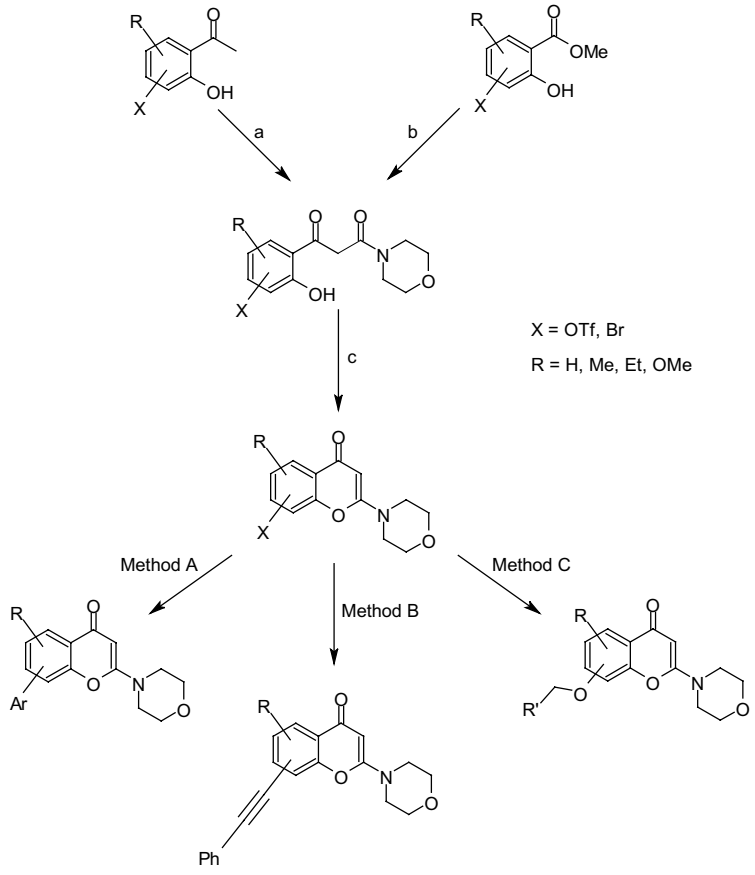
The library of 2-morpholinochromones synthesised is summarised in Table 1 and the general synthetic routes used to prepare them are outlined in Scheme 1. The synthesis of 2-morpholinochromone analogues was

Table 1. Molecular structures and PDE2 inhibition of the 2-morpholinochromones



Compd	R_6	R_7	R_8	Ref.	PDE2 inhibition (% basal) at 50 μM[a]
1	H	H	Ph	10	54 ± 3
2	H	H	(4-F)Ph	10	43 ± 3
3	H	H	(2-CF_3)Ph	10	43 ± 2
4	H	H	(2-CH_3)Ph	10	47 ± 5
5	H	H	(2-CH_3O)Ph	10	47 ± 2
6	H	H	(2-Cl)Ph	10	62 ± 7
7	H	H	(4-PhO)Ph	10	75 ± 3
8	Me	H	Ph	10	60 ± 6
9	Et	H	Ph	10	47 ± 3
10	OMe	H	Ph	10	75 ± 2
11	OMe	H	(2-Cl)Ph	13	78 ± 2
12	OMe	H	(3-Cl)Ph	13	87 ± 2
13	OMe	H	(4-Cl)Ph	13	67 ± 6
14	OH	H	Ph	13	69 ± 4
15	OMe	H	H	12	38 ± 6
16	H	H	OMe	10,12	20 ± 8
17	H	H	OH	10,12	19 ± 11
18	H	H	OCH_2Ph	12	40 ± 4
19	H	H	OCH_2(3-pyridyl)	12	35 ± 4
20	H	H	C≡CPh	12	93 ± 1
21	H	H	$(CH_2)_2Ph$	12	43 ± 7
22	H	OCH_2Ph	Me	11,12	38 ± 4
23	H	OCH_2(3-pyridyl)	Me	11,12	35 ± 2
24	H	$(CH_2)_2$(4-Me-1-piperazinyl)	Me	11	−2 ± 6
25	H	Ph	Me	12	31 ± 14
26	H	C≡CPh	Me	12	44 ± 1
27	H	OCH_2Ph	H	12	12 ± 3
28	H	OCH_2(3-pyridyl)	H	12	22 ± 17
29	H	Ph	H	13	9 ± 7
30	H	C≡CPh	H	13	92 ± 1

[a] All values are mean ± SE ($n \geqslant 3$).



Scheme 1. Reagents: (a) LiHMDS, 4-morpholinecarbonyl chloride, THF; (b) LDA, 4-acetylmorpholine, THF; (c) triflic anhydride, CH_2Cl_2.

performed as described previously.[10–13] In brief, a range of bromo or triflate substituted salicylacetamides were prepared by condensation of appropriately substituted 2′-hydroxyacetophenones with 4-morpholinecarbonyl chloride. Alternatively, salicylate esters were condensed with 4-acetylmorpholine. Cyclodehydration yields the corresponding 2-morpholinochromones. Both the triflate and bromo precursors are amenable to Suzuki cross-coupling reactions[10] (Method A) yielding the biaryl products (**1**–**13**, **25** and **29**). Demethylation[14] of (**10**) using $AlCl_3$/NaI gave the hydroxy compound (**14**). The key triflate intermediates used for Suzuki couplings were also derivatised by the Sonagashira reaction[12] (Method B) to yield the alkyne analogues (**20**, **26** and **30**). Hydrogenation[12] of (**20**) gave the phenethyl substituted compound (**21**). Alternatively, the triflates were readily hydrolysed to the corresponding phenols and alkylated (Method C), to yield benzyloxy compounds (**18**, **22**, **27**) or 3-pyridylmethoxy compounds (**19**, **23**, **28**).[12] The intermediate 7-methyl-8-hydroxy-2-morpholinochromone was also treated with dibromoethane to give a bromoethoxy adduct, which in turn was treated with *N*-methylpiperazine to give the potent antiplatelet compound (**24**).[11]

In the first instance, our interest was in measuring inhibition of phosphodiesterase activity by LY294002. The isozymes were purified from human platelet lysate by anion exchange chromatography according to the general procedure of Dickinson et al.[9] Activity of pooled fractions was determined by assays under optimal conditions for the two isozymes with measurement of the AMP product quantified by reverse phase HPLC.[15] The activity of each isoform was confirmed by the use of selective isoform inhibitors, namely EHNA for PDE2 and milrinone for PDE3.[16] The results are shown in Figures 3 and 4. LY294002 was shown to inhibit the phosphodiesterase activities of both isozymes in a dose dependent manner, but was a significantly more potent inhibitor of PDE2, with an IC_{50} of 40 μM compared with an IC_{50} of 100 μM for PDE3. Under these conditions EHNA was found to have an IC_{50} of 5 μM versus

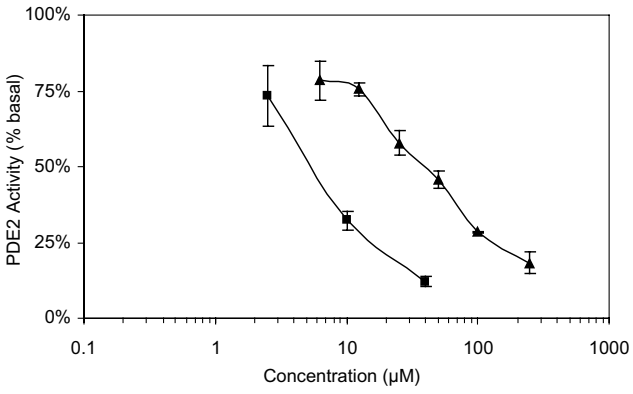


Figure 3. Inhibition of PDE2 activity by EHNA (■) and LY294002 (▲).

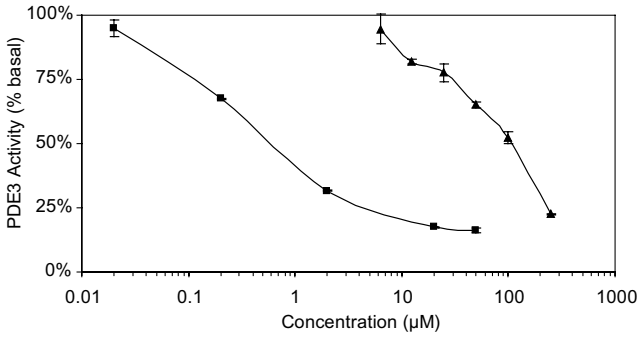


Figure 4. Inhibition of PDE3 activity by milrinone (■) and LY294002 (▲).

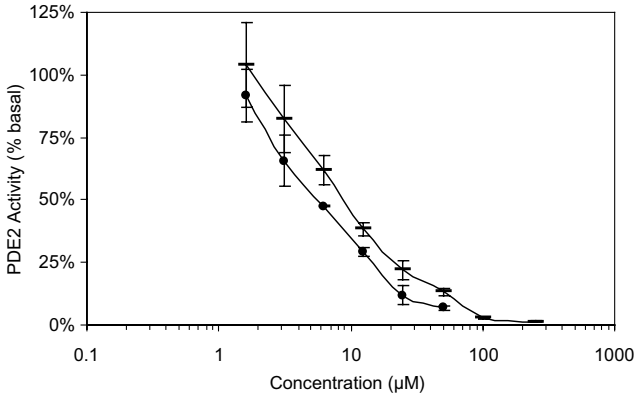


Figure 5. Inhibition of PDE2 activity compounds **12** (—) and **20** (•).

PDE2 while milrinone had an IC_{50} of 0.6 μM versus PDE3, consistent with the reported literature values.[16] This is the first report of phosphodiesterase inhibition by LY294002.

Having detected this level of potency against PDE2 by LY294002, we decided to explore the activity of the 2-morpholinochromone structural class more thoroughly. We screened the compounds of our analogue library at a single concentration of 50 μM. The results of this screen are shown in Table 1. A number of compounds showed strong inhibition of PDE2 activity. Of the LY294002 analogues, which were substituted on the pendant aryl ring, the bulky *para*-phenoxy substituent (**7**) showed 75% inhibition of PDE2 activity while the introduction of *ortho*-substituents on the pendant aryl ring (compounds **3**–**6**) was generally benign. Improved levels of inhibition were generally observed across the range of LY294002 derivatives bearing substituents at the 6-position, with the 6-methoxy-8-(3-chloro)phenyl analogue (**12**) effectively abolishing activity at 50 μM. Both the 8- and 7-phenylethynyl substituted compounds (**20** and **30**, respectively), had the same marked effect of near complete inhibition. The remaining 6-, 7-, 8- and 8-methyl-7-substituted compounds showed inhibitory potency inferior to that of LY294002.

The results from the preliminary screen prompted us to give attention to two of the more potent compounds. The 6-methoxy-8-(3-chloro)phenyl (**12**) and 8-phenylethynyl (**20**) compounds were both found to inhibit PDE2 in a dose dependent manner (Fig. 5). Compound **20** was found to be equipotent with EHNA in this assay, with an IC_{50} of 6 μM, while for compound **12** an IC_{50} of 9 μM was determined.

While LY294002 has been widely used as one of the tools to delineate the role that phosphatidylinositol 3-kinases play in the regulation of intracellular signalling pathways, other properties have been reported for this compound in more recent times. These include inhibition across the broad PI3K-like kinase family, including the serine/threonine kinases DNA-dependent protein kinase (DNA-PK),[17] as well as other kinases such as creatine kinase 2 (CK2).[18] Most recently, LY294002 has been reported to be a potent antagonist of the oestrogen receptor.[19] Our own concerns with LY294002 related to the effects it might be expected to have on the cGMP-inhibited phosphodiesterase (PDE3). In the event, however, we found that LY294002 was a poor inhibitor of PDE3 but a moderate inhibitor of PDE2. The IC_{50} of approximately 40 μM would not generally be enough to compromise the existing literature relating to LY294002 but in some experimental settings LY294002 has been used at high concentrations, in which case caution may be required in interpreting results.

There is some precedent for the overlap of kinase and phosphodiesterase activity beyond 2-morpholinochromones. Natural product flavonoids, such as quercetin, and purines, such as theophylline and caffeine, have been reported to inhibit both PI3Ks and PDEs.[20–22] The planarity of the purine and chromone ring structures would appear to be a fundamental feature of this overlapping activity. As a great deal of effort is being put in to determining the 'kinase selectivity' of many new inhibitors,[18,23] it would seem prudent to include other nucleoside binding targets such as phosphodiesterases and purine receptors in such screens. Indeed, as in this case, kinase-inhibitor libraries might be an excellent resource for discovering new ligands for phosphodiesterases.

The inhibitory activity of LY294002 and the analogues shown in this study may prove to be an excellent lead for the development of PDE2 selective ligands. The results of the screen show three distinct points at which elaboration of the 2-morpholinochromone structure might improve upon inhibitor potency. Firstly, inclusion of a phenethynyl substituent yielded the two most potent inhibitors of PDE2 of this study, with compound **20** confirmed to be equipotent with EHNA. Furthermore, addition of a 6-methoxy substituent to the 2-morpholinochromone backbone of LY294002 was found to enhance activity against PDE2, as was substitution on the pendant aryl ring of LY294002. The inclusions of *para*-phenoxy and *meta*-chloro substituents on the pendant aryl ring were of particular interest. Our preliminary studies suggest these modifications are benign or deleterious to PDE3 and PI3K potency, and studies to determine the broader selectivity of these compounds are underway.

These studies reveal a new structural motif for inhibitors of PDE2. We have shown that inhibition of PDE2 can be improved by relatively simple substitutions and that these compounds display some discrimination between PDE2 and PDE3. Realising the potential of these ligands rests with optimisation of phosphodiesterase activity at the expense of PI3K and other potential activities. Such a goal may yet be difficult to achieve, but the addition of a new small molecule template will greatly assist our understanding of PDE2-inhibitor interactions and lead the way to the development of therapeutic opportunities across a range of diseases.

Acknowledgements

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15. Assay conditions: A typical PDE2 assay consisted of 70 μL of HEPES buffer to which 0.5 μL DMSO or inhibitor, 1 μL 1 mM cGMP (final concentration 10 μM) and 20 μL PDE2 were added followed by mixing in an incubator at 37 °C for 90 s. The enzymatic reaction was initiated by the addition of 10 μL 300 μM cAMP (final concentration 30 μM) and the reaction terminated after 5 min by placing the reaction vessel in an oven at 100 °C for 3 min. Analysis was carried out using reverse phase HPLC on an Agilent 1100 HPLC system, in an adaptation of the method of Ogata, S.; Suzuki, A.; Kawase, T. *Jpn. J. Oral. Biol.* **1995**, *37*, 1. A 50 μL aliquot of the assay reaction was injected onto an Waters Xterra RP C_{18} column equilibrated at 1% acetonitrile in 0.2 M $(NH_4)H_2PO_4$ aq at 0.3 mL/min flow rate. The elution gradient program was (a) 1% acetonitrile isocratic for 3 min, (b) 1–10% acetonitrile linear gradient over 5 min, (c) 10% acetonitrile isocratic for a further 2 min and (d) return to the starting conditions over 1 min and re-equilibration for 10 min. Peak detection was carried out at 254 nm and compared to AMP and cAMP standards. Phosphodiesterase activity was determined as the peak area of AMP (mAU) measurement as a percentage of the DMSO control (where the control without any inhibitor present indicates maximal AMP production under the assay conditions). Unless otherwise stated, all assays were completed at least in triplicate. Data is presented as the mean ± the SE. IC_{50} is defined as the concentration of inhibitor where 50% of the AMP production has been inhibited as compared to the DMSO control.
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